Exhibit 98

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The Role of the Tumor Stroma in Ovarian Cancer

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Abstract

The tumor microenvironment, consisting of stromal myofibroblasts, endothelial cells, and leukocytes, is growingly perceived to be a major contributor to the pathogenesis and disease progression in practically all cancer types. Stromal myofibroblasts produce angiogenic factors, proteases, growth factors, immune response-modulating proteins, anti-apoptotic proteins, and signaling molecules, and express surface receptors and respond to stimuli initiated in the tumor cells to establish a bi-directional communication network in the microenvironment to promote tumor cell invasion and metastasis. Many of these molecules are candidates for targeted therapy and the cancer stroma has been recently regarded as target for biological intervention. This review provides an overview of the biology and clinical role of the stroma in ovarian cancer.

Keywords: ovarian carcinoma, stromal myofibroblasts, metastasis, tumor progression, prognosis

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Introduction

Cancer is characterized by uncontrolled cell growth due to the combined effect of growth-promoting and cell death-suppressing signaling. Tumor growth and progression in carcinomas characteristically involves a pre-invasive phase, followed by invasion of the surrounding stroma, entry into blood and lymphatic vessels, and metastasis. It is growingly perceived that all these phases require cross-talk between tumor cells and their microenvironment, which consists of immune system effectors, endothelial cells, and stromal myofibroblasts. The latter cell population, often referred to as cancer-associated fibroblasts (CAF), has a particularly important role in tumor biology, due to its ability to dynamically modify the composition of the extracellular matrix (ECM), thereby facilitating invasion and subsequent metastatic colonization, and to produce and secrete tumor-promoting factors (1–3). This has impacted on the development of therapeutic strategies designed at targeting stromal myofibroblasts in cancer (4).

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Ovarian cancer, the most lethal gynecologic malignancy (5), is a heterogeneous group of malignant tumors, of which ovarian carcinoma (OC) is the most common one. The common histological types of OC – serous, endometrioid, clear cell, and mucinous carcinoma, are distinct morphological entities that are growingly perceived to be of different etiology, with unique genetic and phenotypic characteristics and different clinical behavior, including response to chemotherapy (6). OC patients are diagnosed with advanced-stage disease in the majority of cases, and despite aggressive surgery combined with platinum-based chemotherapy often succumb to their disease, primarily due to chemoresistance in recurrent tumors (7).

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As in other cancers, the OC stroma produces and expresses myriad molecules relevant for tumor biology, and the mere presence of a large stroma component in OC was reported to be associated with poor survival in advanced-stage disease (8). This review summarizes current data regarding the expression and clinical relevance of molecules related to the cancer microenvironment in OC stromal cells. Data related to the immune system or to the tumor vasculature are not discussed. Studies of areas which remain controversial, such as the role of mesenchymal stem cells in OC biology, are similarly not the focus of this paper.

Proteases

Proteases are critical mediators of invasion and metastasis and are

the cancer-associated molecules which have been most frequently studied in the OC stroma. Studies have predominantly focused on the matrix metalloproteinase (MMP) family, but a significant number of papers have focused on urinary-type plasminogen activator and cathepsin D.

Matrix metalloproteinases are a family of at least 23 membrane-bound (MT-MMP) or secreted zinc-dependent endopeptidases involved in invasion, tumor growth, inflammation, and angiogenesis. MMP family members share several domains, including a signal peptide required for secretion, a propeptide which keeps the enzyme latent, catalytic domain, and hemopexin-like domain, the latter required for binding tissue inhibitors of metalloproteinases (TIMP) and MMP activation. MMP-2 (Gelatinase A, 72 kDa type IV collagenase) and MMP-9 (Gelatinase B, 92 kDa type IV collagenase) additionally contain a collagen-binding area adjacent to their catalytic domain. In addition to ECM molecules, MMP substrates include proteases (other MMPs, plasminogen), growth factors (transforming growth factor; TGF), tyrosine kinase receptors (epidermal growth factor receptor, fibroblast growth factor receptor; EGFR, FGFR1), adhesion molecules (CD44, E-cadherin, αV integrin), chemokines, and the metastasis inhibitor KISS-1. MMPs are negatively regulated by various proteins, including TIMP-1–4, α2 macroglobulins, thrombospondins, and RECK. However, MMP-2 activation requires the formation of a complex with TIMP-2 and MT1-MMP (MMP-14) (9–11).

Collagen I and an anti-β1 integrin antibody induced activation of proMMP-2 in OC-derived fibroblasts *in vitro* (12). OC cell lines implanted in the peritoneal cavity of mice lacking the MMP-9 gene had fewer and smaller tumors than cells injected into mice with wild-type MMP-9 (13). MMP-2, MMP-9, MT1-MMP, and MT2-MMP were detected in the mouse stroma in animals inoculated with OC cells, but only MMP-2 and MT1-MMP levels were increased compared to normal mouse ovaries. Stromal expression of these molecules was unrelated to metastasis, the latter being rather related to tumor MT1-MMP levels (14).

The presence of stromal MMP-1, MMP-2, MMP-9, MT1-MMP, and TIMP-2 mRNA and/or protein has been shown in multiple studies of clinical OC specimens (15–35). However, the clinical significance of MMP and TIMP expression in the OC stroma remains controversial. In analysis of 90 primary OC, MMP-2, MMP-9, and MT1-MMP protein expression in stromal cells by immunohistochemistry (IHC) was significantly related to

advanced-stage disease and poor disease-specific survival (DSS). Stromal MMP-9 and MT1-MMP were independent prognosticators in multivariate analysis (28). Higher stromal MMP-9 protein expression was similarly related to poor DSS in univariate, though not multivariate, analysis in another study (31). Stromal MMP-2 protein expression was related to shorter overall and disease-free survival (OS, DFS) in endometrioid, but not in serous OC in a third report (27). In contrast, in a smaller study of 33 OC, absence of MMP-2 from the OC stroma was associated with more aggressive disease (20). TIMP-2 mRNA expression in stromal cells of both primary OC and OC metastases was associated with poor outcome in univariate analysis, whereas the presence of MT1-MMP mRNA in stromal cells in metastases correlated with significantly longer survival. The association between stromal TIMP-2 mRNA expression in primary carcinomas and poor survival retained its significance in a multivariate analysis. Stromal MMP-2 and MMP-9 mRNA expression in primary or metastatic disease was unrelated to survival (19). In contrast, stromal TIMP-2 protein expression was significantly related to better chemoresponse and longer progression-free survival (PFS) and OS in analysis of 43 tumors (33).

Stromal expression of MMP-2 (30–32, 34), MMP-7 (34), MMP-9 (34), MMP-11 (32), MT1-MMP (34), TIMP-1 (34), and TIMP-2 (34) proteins was unrelated to survival in several studies.

The glycoprotein extracellular matrix metalloproteinase inducer (EMMPRIN; CD147) is member of the immunoglobulin superfamily of adhesion molecules, which stimulates the synthesis of several MMPs and binds MMP-1 and integrins on the surface of tumor cells.

Extracellular matrix metalloproteinase inducer was detected in tumor cells in primary OC, solid metastases, and malignant effusions in OC, as well as in stromal cells and endothelial cells. In solid lesions, EMMPRIN mRNA by *in situ* hybridization (ISH) was significantly co-expressed with β1 integrin mRNA in stromal cells. In survival analysis, EMMPRIN protein expression in stromal and endothelial cells of primary carcinomas correlated with poor survival (36).

Extracellular matrix metalloproteinase inducer protein expression by immunofluorescence was found in both tumor and stromal cells in a study of 120 primary OC and 40 intraperitoneal metastases. The monocarboxylate transporters MCT1 and MCT4, reported to be associated with EMMPRIN expression and drug resistance, were additionally detected in these specimens (37).

Urokinase-type plasminogen activator (uPA) is a serine protease that is synthesized as a latent pro-enzyme and activated by several proteases, including plasmin, cathepsins B and L, and kallikreins (KLKs). uPA and its homolog tissue-type PA (tPA) cleave plasminogen to plasmin, thereby mediating degradation of fibrin and other ECM proteins and the activation of several MMPs, as well as growth factors such as basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), and TGF-β. The uPA receptor uPAR additionally binds ECM proteins and integrins. The plasminogen activator inhibitors PAI1 and PAI2 and the plasmin inhibitor α2 antiplasmin negatively regulate this system (38, 39).

Analysis of uPA mRNA and protein expression in 57 ovarian tumors and 8 abdominal metastases showed expression of uPA mRNA in epithelial cells in benign and borderline tumors, whereas poorly differentiated primary OC and metastases of different histological grade had predominantly stromal expression. In contrast, uPA protein expression was seen in both compartments (40). In another paper by this group, increased expression of uPA, uPAR, and PAI1 mRNA was found in poorly differentiated primary OC with solid growth pattern and in metastases compared to cystic, better differentiated tumors (41). Protein expression of uPA and uPAR, as well as several MMP members, was frequently seen in the OC stroma in both primary carcinomas and metastases, though uPA and uPAR were absent in the stroma of well-differentiated tumors (42). In a murine OC model, uPAR$^{-/-}$ mice lacking uPAR in host mesothelial cells had reduced tumor and ability to form peritoneal metastases, as well as reduced ascites formation and longer survival compared to uPAR$^{+/+}$ mice. In clinical specimens, higher stromal uPAR protein expression was seen in OC compared to normal ovaries, with higher expression associated with higher histological grade (43).

The ETS family of transcription factors regulates the transcription of a large number of cancer-associated molecules, including uPA, uPAR, MMP-7, and MMP-9, as well as the apoptosis inhibitor Survivin, the tumor suppressor Maspin, the cell cycle protein p21/CIP1, and Slug, mediator of epithelial-to-mesenchymal transition (EMT), thereby affecting many cellular processes, including angiogenesis, invasion and metastasis, and cell survival (44).

Ets-1 mRNA is co-expressed with MMP-1 and MMP-9 mRNA in the OC stroma (22). In analysis of 66 primary and metastatic OC from long-term and short-term survivors, Ets-1 mRNA was

detected in stromal cells in 33% of cases using ISH (Figure 1), more often in tumors of short-term survivors, and was co-expressed with vascular endothelial growth factor (VEGF) mRNA. Ets-1 mRNA expression in both tumor and stromal cells was associated with poor survival in univariate analysis, and expression in stromal cells was an independent prognostic factor in a multivariate analysis (45).

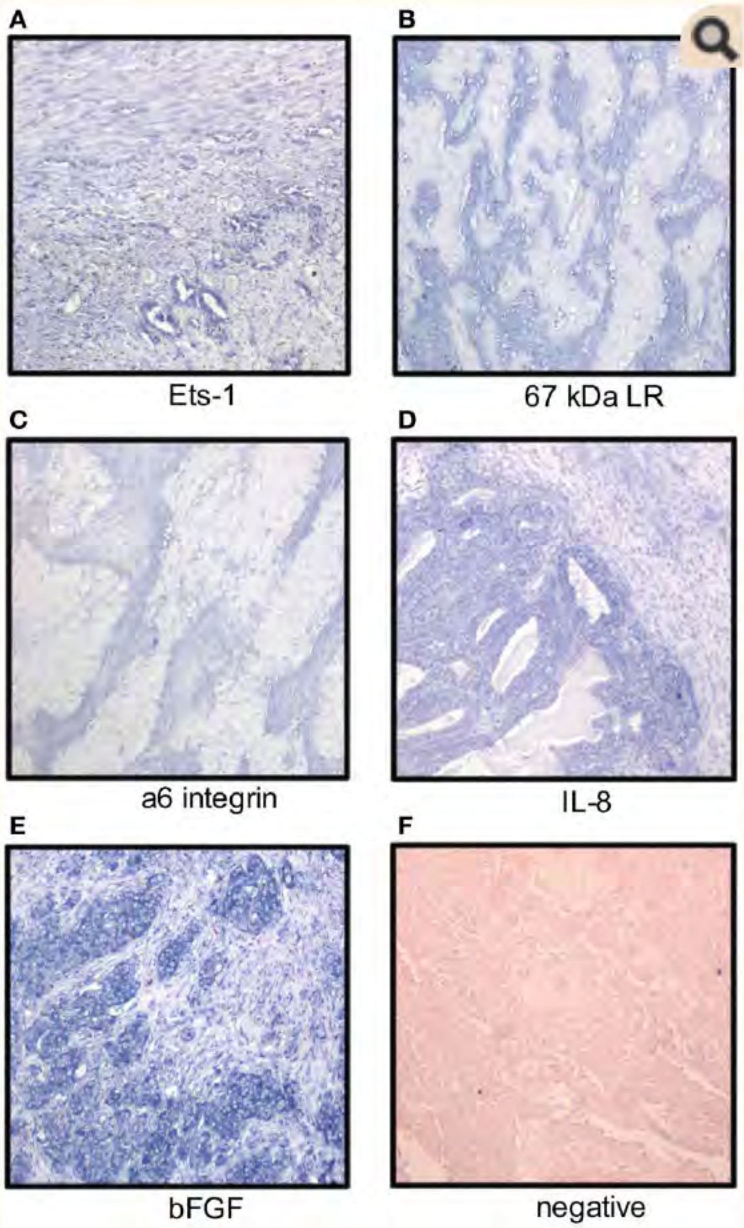

Figure 1

Localization of mRNA of cancer-associated molecules to the ovarian carcinoma stroma. OC stromal cells express mRNA of the Ets-1 transcription factor **(A)**, laminin receptors **(B,C)**, and the angiogenic factors IL-8 and bFGF **(D,E)**; **(F)** negative control. Tumor cells express Ets-1, IL-8, and bFGF (NBT-BCIP as

chromogen, nuclear fast red as counterstain).

In another study of the same cohort, the expression of PEA3, another Ets family member, was assessed using ISH. PEA3 mRNA was detected in stromal cells in 89% of tumors, but strong expression was limited to the stroma of grade 2–3 tumors. PEA3 mRNA expression in stromal cells was significantly related to MMP-2 mRNA expression in carcinoma cells, whereas PEA3 expression in carcinoma cells was significantly related to mRNA expression of the β1 integrin subunit, bFGF, and EMMPRIN in stromal cells. PEA3 mRNA was detected significantly more often in both carcinoma and stromal cells in tumors of short-term survivors and PEA3 expression in stromal cells correlated with shorter DFS and OS in univariate and multivariate survival analysis (46).

The clinical role of cathepsins, another family of proteases, was investigated in several studies. The level of cathepsin D, a lysosomal aspartyl protease, measured by immunoradiometric assay in OC tissue homogenates, was unrelated to clinical parameters or survival, with similar results for protein expression in tumor and stromal cells by IHC (47). In a study limited to stage III tumors ($n = 185$), tumor cell cathepsin D expression was related to longer OS in univariate analysis, with no such role for stromal expression. However, combined epithelial and stromal expression was an independent prognostic factor in multivariate analysis (48). No association was found with PFS. In contrast, cathepsin D expression in stromal cells was an independent prognostic factor of longer DFS, but not OS, in IHC analysis of 80 OC, with no prognostic role observed for tumor cell expression (49).

Cathepsin B, a cysteine protease, and the cysteine protease inhibitor cystatin C were detected in OC cells and their stroma, and were absent in cystadenomas (50).

Tissue KLKs are a family of 15 serine proteases encoded by a single gene cluster located at chromosome 19q13.4. Analysis of KLK4 expression in 43 primary and 63 metastatic OC showed stromal KLK4 expression in 48/103 specimens, which was significantly higher in primary tumors compared to metastases, with no prognostic role for this protein (51).

ECM Proteins and Their Receptors

The ECM composition in OC and its clinical relevance has been the

subject of several studies.

Analysis of mRNA expression of the proα1(I) and proα2(I) chains of type I procollagen and of the proα1(III) chain of type I procollagen by ISH demonstrated their localization to the OC stroma, whereas expression was weaker or absent in the stroma of benign cysts. In poorly differentiated carcinomas ($n = 2$), signals were additionally detected in tumor cells (52). Differences in the density of collagen type I fibers were observed between cystadenomas, borderline tumors, and OC of different histological grade in another study (53). Oncofetal fibronectin was detected in the OC stroma, but not in endometriosis, suggesting this protein was selectively expressed by the tumor microenvironment (54). Fibulin-1, an estrogen-regulated calcium-binding and acidic ECM glycoprotein, was localized to the OC stroma, with strongest expression in proximity to tumor cells, and its mRNA was localized to the latter compartment. Staining increased from normal ovaries through benign and borderline tumors to OC, and was associated with progesterone receptor, but not estrogen receptor expression (55).

Analysis of the expression pattern of laminin γ2 chain in mucinous ovarian tumors with gastrointestinal differentiation by IHC showed basement membrane localization in adenomas, borderline tumors, intraepithelial carcinomas, and adenocarcinomas with expansile growth pattern, whereas expression was cytoplasmic or stromal in carcinomas growing with infiltrative pattern (56). Stromal expression of laminin-5 γ2 chain with concomitant presence of MT1-MMP on the tumor cell surface was reported in clear cell OC (57). Galectin-1, a laminin-binding protein regulating tumor cell proliferation and adhesion to matrix, was overexpressed in OC compared to normal ovaries and co-localized with laminin-1 and fibronectin. Its levels were increased in fibroblasts cultured with OC cells *in vitro* with effect on tumor cell proliferation and adhesion (58). Analysis of the expression of two laminin receptors, the 67-kDa laminin receptor precursor (LBP) and the α6 integrin subunit, in 41 primary OC and 75 solid metastases showed mRNA expression by ISH in stromal cells in 68 and 20% of cases (Figure 1), respectively. No association with clinicopathologic parameters or outcome was found (59).

Analysis of additional integrin subunits in primary OC and solid metastases showed stromal expression of the β1 integrin subunit mRNA by ISH in 2 independent tumor series, whereas the αV subunit mRNA was found in the stroma in only one of the series. While tumor αV subunit mRNA expression was associated with

poor survival in one of these studies, the presence of these subunits in stromal cells had no prognostic value (60, 61).

The mRNA expression of angiogenic cytokines and growth factors was analyzed in two studies. bFGF, interleukin-8 (IL-8), and VEGF mRNA was expressed in both tumor and stromal cells with no significant difference between primary carcinomas and metastases. bFGF was the most strongly and frequently expressed transcript in primary OC and in solid metastases in both series, with intermediate expression of IL-8 and low expression of VEGF (Figure 1). None of these factors was related to clinicopathologic parameters or disease outcome (62, 63). In another series, IL-8 mRNA expression was higher in tumor compared to stromal cells in OC specimens, whereas the protein was expressed in both compartments. IL-8 receptor B, but not A, was expressed in stromal cells (64). In a study of FGF-8 expression in OC, this cytokine was localized to tumor cells, whereas its receptors FGFR1, FGFR2, and FGFR4 were expressed by tumor cells, and to lesser extent, in stromal cells (65).

Hyaluronan (also termed hyaluronic acid or hyaluronate; HA), a large, linear, negatively charged polysaccharide with strong capacity to attract water, maintains tissue hydration and osmotic balance under normal condition. It additionally regulates cell adhesion, migration, apoptosis, and proliferation via interaction with specific cell surface receptors, which include the adhesion molecule CD44. HA has been shown to be involved in tumor progression of multiple cancers, through its effect on the above processes, as well as angiogenesis, invasion, and EMT (66).

HA is expressed in the stroma of both stage I and stage III OC, and its expression is increased in peritoneal metastases from patients with stage III disease compared to primary carcinomas (67). Analysis of 309 primary OC showed significant association between stromal HA expression and high histological grade, serous histology, advanced-stage and large residual disease volume, with no relationship to tumor cell CD44 expression. High stromal HA expression was further significantly related to poor relapse-free survival (RFS) and OS, and HA was more highly expressed in 45 patient-matched metastases additionally studied (68). Allelic imbalance at chromosome 3p21.3, a region harboring the hyaluronidase genes *HYAL1-3*, was found in microdissected tumor and stromal cells of borderline tumors and OC (69).

The unique stroma of clear cell OC was reported to contain both HA and collagen type IV, and these components were involved in its formation or modification (70, 71).

Proteoglycans, composed of a core protein to which glycosaminoglycan chains are attached, are a family of highly conserved macromolecules localized to the cellular membrane or the ECM. Proteoglycans are expressed by multiple cancers and mediate angiogenesis, tumor growth, invasion, and metastasis (72, 73).

Davies et al. analyzed the expression of syndecan-1–4, glypican-1, and perlecan in 147 ovarian specimens, including 115 OC, using IHC. Syndecan-1 was expressed in tumor and stromal cells of benign ovarian tumors, borderline tumors, and OC, with most intense staining in areas of invasion in OC, and was absent in normal ovaries. Syndecan-2 and -3 and glypican-1 were expressed in the stroma of all types of specimens, as was true for syndecan-4 in epithelial cells. Stromal perlecan expression was frequently seen in benign tissue and borderline tumors, but was lost in 67% of carcinomas. Stromal syndecan-1 expression was significantly associated with poor PFS and OS, though not independently (74).

In another study, stromal syndecan-1 and versican expression were associated with advanced-stage, serous histology, massive ascites, positive peritoneal cytology, and sub-optimal cytoreduction, as well as poor PFS and OS, though not independently (75). Ghosh et al. reported on overexpression of versican in OC compared to normal ovaries, as well as in advanced-stage compared to early-stage disease. Stromal versican expression was associated with higher microvessel counts, platinum resistance, and poor PFS and OS in univariate analysis (76). In another study, stromal versican expression was related to non-mucinous histology, advanced-stage, and reduced 5-year survival rate (77).

Decorin protein was reported to be expressed by the OC stroma, whereas tumor cells were negative, despite the presence of its mRNA in both cellular compartments (78). Periostin was overexpressed in the OC stroma compared to borderline and benign tumors and its presence in OC was associated with advanced-stage, disease recurrence, and poor OS, the latter also in multivariate analysis (79).

TGF-β is a ubiquitous cytokine with a dual role as both growth suppressor and promoter, effects which are largely mediated by the stroma and immune system. TGF-β acts predominantly as tumor promoter in several cancer types, including OC, and is consequently under consideration as a potential therapeutic target (80).

Comparative analysis of TGF-β1 and latent TGF-β1 binding protein 1 (LTBP-1) expression in serous and mucinous OC and adenomas showed strong stromal expression of these proteins limited to the former group (81). Transcriptome analysis of microdissected tumor and stromal cells from OC specimens and TGF-β-treated normal ovarian fibroblasts recently identified versican as an upregulated gene in CAF, and versican expression was upregulated by TGF-β, with resulting activation of the NF-κB signaling pathway and increased levels of CD44, MMP-9, and the hyaluronan-mediated motility receptor (82). Chloride intracellular channel 4 (CLIC4) was shown to mediate conversion of fibroblasts to myofibroblasts following stimulation with TGF-β1 *in vitro* and was frequently expressed in the OC stroma (83). Expression of TGF-β in the stroma of primary and recurrent OC was reported in another study (84).

Protein expression of the βA-subunit of activin A, member of the TGF-β superfamily, which regulates migration and invasion during EMT, metastasis, and MMP expression, was increased in stromal cells from OC specimens compared to adenomas (85).

Stromal protein and mRNA expression of secreted protein, acidic and rich in cysteine (SPARC; a.k.a osteonectin), a matricellular protein involved in angiogenesis and tumor invasion, was higher in OC compared to normal ovaries and borderline tumors. Tumor cells expressed SPARC protein, but not mRNA (86, 87).

Endothelins, mitogenic peptides with autocrine and paracrine effect, stimulated the growth of fibroblast cell lines isolated from ascites specimens of OC patients, and were found in both the tumor cell and stromal compartments in clinical specimens (88).

The platelet-derived growth factor receptors PDGFRα and PDGFRβ were expressed in stromal cells in 32 and 44% of OC in analysis of 170 tumors, but their expression was unrelated to clinical parameters or survival (89).

The granulin–epithelin precursor (GEP/progranulin/PC-cell-derived growth factor) is a 68-kDa secreted protein with several higher molecular weight forms due to glycosylation, most commonly of 88 kDa. GEP was shown to be a growth factor in OC (90). Analysis of 189 solid OC specimens (64 primary OC, 125 metastases) showed GEP expression in stromal and endothelial cells 52 and 67% specimens, respectively. Stromal GEP expression was significantly lower in metastases sampled during or following chemotherapy compared to chemo-naïve tumors, and the presence of GEP-positive stromal cells in untreated primary tumors

correlated with worse OS (91).

Insulin-like growth factor-1 was detected in the OC stroma, with strongest expression around vessels, with less frequent and weaker expression in tumor cells (92).

Transcriptional Regulators

HOX transcription factors constitute a large family of proteins that regulate embryogenesis and organogenesis via spatial cues, as well as by regulating apoptosis, proliferation, differentiation, motility, and angiogenesis. HOX members are differentially expressed in adult tissues and regulate the expression of cadherins, integrins, NCAM (CD56), and p53. Deregulation of HOX members has been shown in different cancers (93, 94).

HOXA7 was overexpressed in the tumor cell nuclei and in the stroma of clear cell OC compared to other OC histotypes, and expression was lowest in serous OC (95). HOXA9 expression in OC cells induced normal peritoneal fibroblasts and adipose tissue- and bone marrow-derived mesenchymal cells to develop CAF features, a process shown to be mediated by TGF-β2 upregulation of CXCL12, IL-6, and VEGF-A (96). HOXA10 expression in OSE cells stimulated interaction with the ECM proteins fibronectin and vitronectin, with omental mesothelial cells and fibroblasts (97).

DNA topoisomerase IIα (TOP2α), an enzyme involved in DNA replication, RNA transcription, chromosomal condensation, and mitotic chromatid separation, is the target of chemotherapeutic drugs such as etoposide and doxorubicin. Comparative analysis of primary and recurrent OC specimens showed reduced TOP2α expression in tumor cells in the latter group, whereas stromal expression was increased (98).

Vestigial like 3, a putative tumor suppressor, was expressed in high-grade serous OC cells, and to a lesser extent in stromal cells, in a series of 182 tumors, and higher stromal expression was associated with a trend for longer survival (99).

Nuclear expression of Snail1, one of the key regulators of EMT, was observed in tumor and stromal cells in 23 and 24% specimens, respectively, in a series of 74 OC. Snail1 expression was minimal in borderline tumors and absent in adenomas and normal ovaries. Snail1 tumor cell and stromal expression was unrelated to clinicopathologic parameters or survival (100).

Expression of two of four studied members of the CCAAT/enhancer binding protein (C/EBP) family of transcription

factors, reported initially to regulate adipocyte proliferation and differentiation, was observed in the OC stroma, whereas all four proteins (C/EBP-α, -β, -δ, and -ζ) were expressed in tumor cells (101).

Nuclear expression of adrenal 4-binding protein/steroidogenic factor-1 (Ad4BP/SF-1) and dosage-sensitive sex reversal adrenal hypoplasia congenita critical region on the X chromosome gene 1 (DAX-1), nuclear receptor superfamily members involved in the regulation of steroidogenesis, was shown in stromal cells in OC. Enzymes involved in ovarian steroidogenesis, including steroidogenic acute regulatory protein (StAR), P450 side chain cleavage enzyme (P450scc), and 3-beta-hydroxysteroid dehydrogenase (3b-HSD) were detected in the stromal cell cytoplasm (102). Stromal protein expression of PPAR-β, another nuclear receptor superfamily member, was reduced in OC compared to borderline tumors, benign tumors, and normal ovaries, whereas expression of its target protein 3-phosphoinositide-dependent protein kinase 1 (PDK1) was limited to epithelial cells and increased in OC (103).

Other Molecules

Various molecules related to other biological pathways have been localized to the OC stroma and are discussed in this section.

Immune response effectors

Several studies have investigated the expression of molecules related to the immune response in OC stromal cells. Proteins reported to be expressed by stromal cells include IL-11 receptor (104), the pro-inflammatory peptide LL-37 and its precursor human cationic antimicrobial protein-18 [hCAP-18; (105)], lymphotoxin-β receptor and the chemokine CXCL11 (106), and CD277 (107), as well as IL-6, COX-2, and CXCL1 (108). The clinical role of these biomarkers in this cellular compartment remains to be established.

IL-1β was recently reported to suppress nuclear p53 expression in CAF. High IL-1β and its receptor IL-1R1 and low p53 expression in CAF were associated with poor OS. p53 knockdown in ovarian fibroblasts resulted in increased expression and secretion of IL-1β, IL-6, IL-8, VEGF, and growth-regulated oncogene-α (GRO-α) and increased tumor growth *in vivo* in a NF-κB-dependent manner (109). Induction of senescence in fibroblasts by GRO-α was previously reported to mediate tumor promotion in a previous study by the same group (110).

Ribonuclease-2 (RNASET2), an extracellular RNase expressed in the OC stroma, was shown to mediate recruitment of macrophages to the tumor microenvironment and its silencing enhanced tumor growth of OVCAR-3 cells *in vivo*. Genes altered following *RNASET2* silencing were involved in pathways related to the immune response and cell adhesion (111).

Cell cycle and apoptosis-related proteins

Protein expression of the cell cycle inhibitor p16 in stromal cells was reported to be associated with improved prognosis, whereas the presence of this protein in tumor cells was a poor prognostic marker (112). Stromal expression of another cell cycle inhibitor, p27, was significantly reduced in OC compared to normal ovaries, as was the expression of lung resistance protein (LRP), a protein associated with multidrug resistance (MDR), whereas multidrug resistance protein (MRP) expression was not significantly different (113). Expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and the death receptors DR4, DR5, and DcR1 was found in OC stromal cells (114). TRAIL was detected in the OC stroma in an additional study (115).

Various molecules

The RNA-binding protein HuR and COX-2 were expressed in the OC stroma in 24 and 7% of specimens in a study of mucinous OC, with no clinical role observed for expression in this cellular compartment (116). Analysis of proteins related to the prostaglandin synthesis pathway using IHC showed expression of COX-2, microsomal prostaglandin E synthase-I (mPGES-I), and the prostaglandin E_2 receptors EP_1 and EP_2 to the OC stroma, particularly in tumors of higher histological grade (117).

Expression of the α, β, and π sub-types of the detoxification enzyme glutathione *S*-transferase was observed in the stroma of OC specimens and different benign tumors (118).

Somatostatin and its receptors sst_1, sst_2, sst_3, and sst_5 were expressed with variable frequency in OC tumor cells and in their surrounding stroma, as well as in the stroma of different benign conditions. Somatostatin was significantly co-expressed with sst_1, sst_2, and sst_5 in the stromal compartment in analysis of the entire cohort (119).

The serotonin receptors 5-HT1A, 5-HTA2, 5-HT2B, and 5-HT4 were expressed, to variable extent, in the stroma of normal ovaries, benign ovarian tumors, borderline tumors, and OC specimens, with

5-HT2B being the most expressed receptor (120).

Retinoic acid receptor-α was found in stromal fibroblasts, tumor-infiltrating lymphocytes, and OC cells in analysis of 16 tumors of serous or mixed histology (121).

Neural endopeptidase (CD10) was expressed in the stroma of serous borderline tumors and in OC of different histotype, whereas no staining was observed in mucinous borderline tumors, in benign tumors, and in normal ovaries (122).

Luteinizing hormone receptor mRNA expression analysis by RT-PCR and ISH was reduced in both tumor cells and the OC stroma compared to benign tumors, with intermediate levels for borderline tumors. Expression in grade 2–3 tumors was less frequent then in their grade 1 counterparts, and the receptor was absent in five analyzed metastases (123).

The expression of six different isozymes of aldehyde dehydrogenase, an enzyme implicated in stem cell biology in OC, was investigated in normal ovaries, adenomas, borderline tumors, and OC specimens. Stromal and tumor cell expression of several isozymes was found to differ between normal tissue and ovarian tumors, as well as between OC of different histotype (124).

Expression of class III β-tubulin was reduced, though not significantly, in the OC stroma following neoadjuvant chemotherapy in analysis of 22 paired tumors obtained pre- and post-chemotherapy. Tumor and stromal class III β-tubulin expression was associated with poor OS (125).

Graphical illustration linking molecules known to have biological association, including HA, bFGF, MMP members, uPA, ETS transcription factors, HuR, and HOXA is shown in Figure 2.

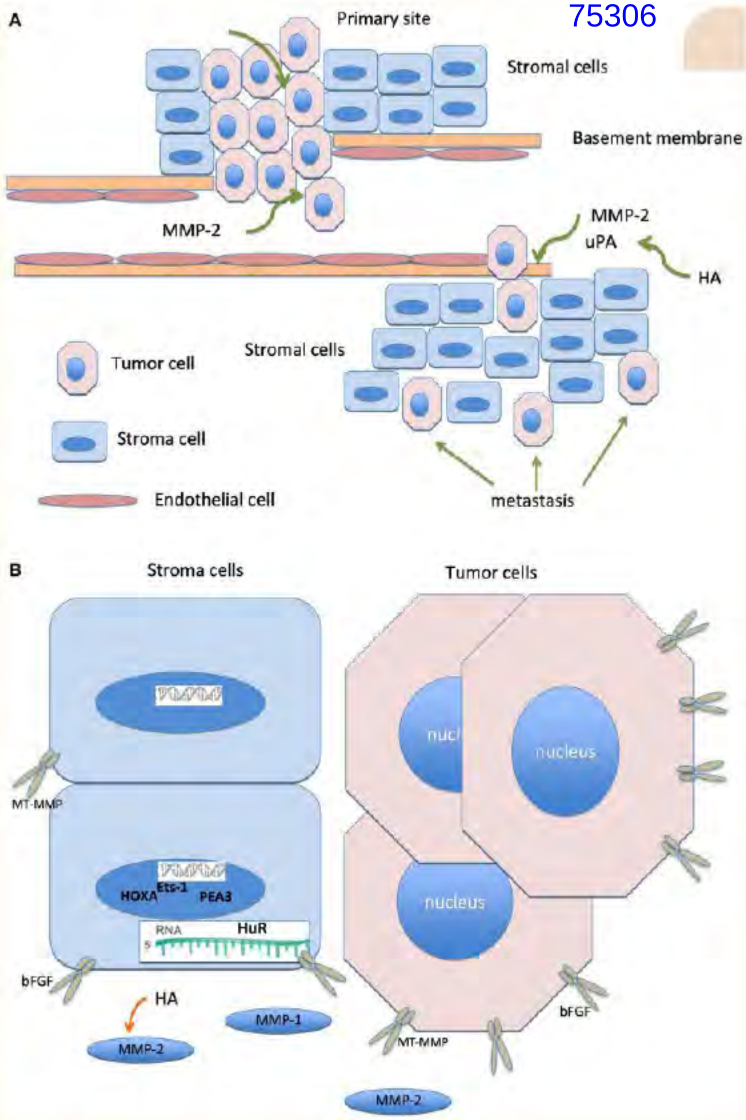

Figure 2

Biologically linked cancer-associated molecules in ovarian carcinoma cells and the tumor stroma. Graphical illustration linking molecules known to have biological association in this cancer, including hyaluronic acid (HA), basic fibroblast growth factor (bFGF), matrix metalloproteinases (MMP), urinary-type plasminogen activator, ETS transcription factors, HuR, and HOXA.

Concluding Comments

Ovarian carcinoma is a highly lethal cancer characterized by considerable heterogeneity across different histological sub-types, as well as within the same morphological entity. In order to achieve noticeable improvement in the outcome of this disease, better understanding of the microenvironment of this tumor at both the primary site and metastatic locations is critically in need.

The above-discussed papers provide compelling evidence regarding the synthetic capacity of CAF in OC and emphasize the cross-talk between tumor cells and the stromal compartment; the latter interaction recently demonstrated *in vitro* (126). They additionally highlight the fact that the clinical relevance of a given molecule may be different or even opposite when expressed in carcinoma cells or in stromal cells. Nevertheless, many of these studies constitute single reports of the expression and clinical role of a given molecule, which need to be confirmed in series from other institutions, preferably studies in which each of the histological types of OC is studied separately.

Recent studies have applied high-throughput technology to the identification of central regulatory pathways in OC fibroblasts, often following microdissection, which allows for analyses focused on the target cell population. Qiu et al. studied genome-wide copy number and loss of heterozygosity (LOH) in CAF isolated from 25 OC and 10 breast carcinoma samples using SNP arrays. LOH and copy number alterations were rarely observed (127). Microarray analysis of microdissected stroma from 24 OC identified 52 candidate genes related to PFS, of which early growth response 1 (*EGR1*) and FBJ murine osteosarcoma viral oncogene homolog B (*FOSB*) were validated in an independent series of 50 tumors and found to be independent prognostic markers of poor PFS (128).

The role of miRNAs in reprograming of normal fibroblasts into CAF through downregulation of miR-31 and miR-214 and upregulation of miR-155 was recently shown, and the chemokine CCL5 was identified as target of miR-214, suggesting a role in modulation of the tumor microenvironment (129).

Exosomes are 30–100 nm lipoprotein vesicles containing proteins, mRNAs, and miRNAs that are secreted from cells and present in most circulating body fluids (130). Exosomes from SKOV-3 and OVCAR-3 cells induced adipose tissue-derived stem cells to acquire characteristics of myofibroblasts, with activation of the TGF-β pathway (131).

Lili et al. studied the stroma of 45 OC by microarray analysis and found two distinct signatures for the stromal compartment, characterized by different pairs of receptors and ligands (132).

Many of the molecules discussed in this review are expressed by both tumor and stromal cells and thereby present the possibility to target both cellular components in order to maximize the tumor-suppressive effect. While clinical studies aimed at inhibiting some of these cellular targets, e.g., proteases and COX-2, have been

largely disappointing, other pathways, particularly receptor tyrosine kinase-driven pathways mediating angiogenesis and other tumor-related processes, are highly relevant (133, 134).

Therapeutic approaches are likely to focus to a larger extent on the tumor stroma in the future, as in the recent study by McCann and co-workers, in which inhibition of *Gli1*, part of the Hedgehog pathway, using the cyclopamine derivative IPI-926 in combination with chemotherapy was assessed (135). Whether such approaches could change the clinical course of OC is yet to be determined.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Exhibit 99

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RESEARCH ARTICLE

Establishment of five immortalized human ovarian surface epithelial cell lines via SV40 T antigen or HPV E6/E7 expression

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Abstract

Background

Human ovarian surface epithelial (HOSE) cells are a critical cell source for ovarian cancer research; however, they are difficult to obtain and maintain under standard laboratory conditions in large quantities. The aim of this study was to generate immortalized HOSE (IHOSE) cells with maintained properties to the original cell source, thereby guaranteeing a sufficiently large cell quantity for ovarian cancer research.

Methods

HOSE cells isolated from four non-cancer patients and five IHOSE cell lines were established by induction of HPV-E6/E7 expression or SV40 large T antigen using a lenti-viral system. Each of IHOSE cells was confirmed to be distinct by STR profiling. RNA-sequencing was used to compare gene expression profiles in HOSE, IHOSE and ovarian cancer cells.

Results

RNA-sequencing results revealed a stronger linear correlation in gene expression between IHOSE and HOSE cells ($R^2 = 0.9288$) than between IHOSE or HOSE cells and ovarian cancer cells ($R^2 = 0.8562$ and $R^2 = 0.7982$, respectively). The gene expression pattern of 319 differentially expressed genes revealed minimal differences between HOSE and IHOSE cells, while a strong difference between ovarian cancer cells and HOSE or IHOSE cells was observed. Furthermore, the five IHOSE cell lines displayed morphological characteristics typical of epithelial cells but showed a lower level of EpCAM, CD133 and E-cadherin, as cancer stem marker, than ovarian cancer cells. Moreover, unlike cancer cells, IHOSE cells could not form colonies in the anchorage-independent soft agar growth assay.

Conclusion

These findings demonstrate that five newly established IHOSE cell lines have characteristics of progenitor HOSE cells while exhibiting continuous growth, and thus, should be highly useful as control cells for ovarian cancer research.

Introduction

Ovarian cancer has a poor prognosis with the lowest survival rate among all gynecological cancers, which is mainly due to the lack of early symptoms, resulting in diagnosis when the cancer has already progressed to an advanced stage [1]. The World Cancer Report of the International Agency for Research on Cancer stated that 114,240 women were diagnosed with ovarian cancer in 2014, with a 5-year survival rate below 45% [2]. In the United States, the mortality rate of ovarian cancer ranks fifth among all cancer patients, with 22,440 new patients with ovarian cancer diagnosed in 2017 resulting in 14,080 deaths [1]. Improvement of this situation requires more extensive research on epithelial ovarian cancer, which necessitates an adequate quantity of human ovarian surface epithelial (HOSE) cells as controls for comparisons of the specific properties and biological behaviors of ovarian cancer cells. However, HOSE cells have an extremely short life span in monolayer cell culture, which has thus far limited ovarian cancer research. Although culture of HOSE cells in a modified medium (NOSE-CM) could potentially prolong cell survival compared to culture in more common media [3], this method alone cannot sustain the amount of HOSE cells required for basic research purposes. Therefore, cell immortalization methods that allow continuous cell growth without limitation of cellular life span have been actively investigated [4±7], including viral gene induction that controls proteins involved in the cell cycle and artificial expression of core proteins related to cell immortality [8]. Specifically, immortalized cell lines are established by overexpression of the HPV-E6/E7 protein or SV40 T antigen in healthy ovarian surface epithelial cells [4, 5]. Alternatively, overexpression of human telomerase (hTERT) instead of HPV-E6/E7 has been reported to maintain cellular functions of pRB and p53 [6]. Moreover, the success rate of producing immortalized cell lines increases when hTERT overexpression is coupled with overexpression of HPV-E6/E7 or SV40 T antigen compared to overexpression of hTERT alone [7]. Furthermore, once an immortalized cell line is established, it must be verified by confirming that the characteristics of the progenitor cell line are preserved. For an epithelial cell line, such observations are based on examination of the cellular morphology and expression pattern of the epithelial marker cytokeratin [9]. In addition, any changes in chromosomes that may have been induced by the immortalization protocol are screened by karyotype analysis [10] and/or the presence of gene mutations from the progenitor cell using whole-exome sequencing [11]. Actually, ovarian cancer has been known to originate from the ovarian surface epithelium (OSE) since the mid-90s to early 2000s [12±15]. To understand the ovarian carcinogenesis, immortalized OSE (IOSE) cells were constructed by the overexpression of immortalized SV-40 T antigen, telomerase and the HPV E6/E7 protein by various study groups [12±14, 16±20]. Several studies have been attempted to identify the genetic differences and their functions in IOSE cells as an intermediate step in cancer, in order to understand the function of pre-malignant or tumorigenic cells [12±15, 17, 21]. In Clinical Cancer Research article (2003), the differences in the gene expression between the IOSE and normal OSE cells were compared using microarray, and whether IOSE cells could be used as a control for ovarian cancer research was discussed [19]. Since then, in the late 2000s and mid-2010, an IOSE cell line was used as an experimental control for ovarian cancer, and these cells were used as a tool to identify the functions and mechanisms of genes implicated in cancer cells [20, 22±28]. Although it has been concluded that the origin of serous ovarian carcinoma arises from the fallopian tube epithelium, and that the endometrioid and clear cell carcinoma are derived from endometriosis [29±31], IOSE cells are still used in many studies as an experimental control and to understand gene functions. Moreover, various kinds of immortalized cells generated in the future might serve as important experimental tools [26±28]. Here, we used an RNA sequencing technique for gene expression profiling to verify whether our established immortalized HOSE (IHOSE) cells

retained characteristics of the progenitor HOSE cells in comparison to those of ovarian cancer cells. In addition, we investigated whether the IHOSE cells demonstrated any malignant features of cancer cells based on cancer stem cell marker expression and an anchorage-independent growth assay. Confirmation of these characteristics and stability of IHOSE cells established with the proposed method could provide a useful resource for comparative or gene expression studies on ovarian cancer toward identification of novel therapeutic and/or diagnostic targets.

Materials and methods

Cell culture

HOSE cells were obtained by scraping the surfaces of healthy ovaries from patients without cancer, and were provided by the Korea Gynecologic Cancer Bank through the Bio&Medical Technology Development Program of the Ministry of the National Research Foundation (NRF) funded by the Korea government (MSIT) (NRF-2017M3A9B8069610). The monolayer of HOSE cells was cultured with M199/MCDB basal medium (1:1) supplemented with 10% Fetal bovine serum FBS and 1% penicillin/streptomycin. HOSE cells were maintained up to six passages, but most HOSE cells aged at two or three passages. IHOSE cells were established by transfecting HPV E6/E7 and SV40 T antigen to short-cultured HOSE cells using a lentiviral system. Cells were grown in DMEM containing 10% FBS with 1% penicillin / streptomycin and cultured at 37ÊCin 5% CO_2. Images of the cells were acquired using an Cell Imaging System (Thermo Fisher Scientific, Rockford, IL). This study was approved by the institutional review board of Gangnam Severance Hospital, and informed consent was obtained from each patient before sample collection. All cell lines were established in the Laboratory of Obstetrics and Gynecology, Gangnam Severance Hospital, Seoul, Korea. Five IHOSE cell lines were deposited with the Korean Gynecology Cancer Bank (KGCB), and are available to researchers. SKOV3 and OVCAR3 cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). SKOV3 and OVCAR3 cell line was maintained in RPMI-1640 supplemented with 1% penicillin / streptomycin and were cultured at 37ÊCin 5% CO_2.

Lentiviral production and infection

To generate pCDH-HPV-E6 and pCDH-HPV-E7, cDNA encoding HPV-E6 or HPV-E7 was amplified from total RNA of cervical cancer SiHa cells using the following primer sets for `HPV-E6: 5′-AAGAATTCATGCACCAAAAGAGAACTGCAAT-3′ (forward) and 5′-AAGGATCCTTACAGCTGGGTTTCTCTACGTG-3′ (reverse), and HPV-E7: 5′-AAGAATTCATGCATGGAGATACACCTACATT-3′ (forward) and 5′-AAGGATCCTTATGGTTTCTGAGAACAGATGG-3′ (reverse).` The amplified cDNA was cloned into EcoRI and BamHI restriction sites of the pCDH-EF1-MCS-T2A-copGFP Lentivector (System Biosciences, Mountain View, CA). pLenti CMV/TO SV40 small+Large T vector was obtained from Addgene (Cambridge, MA). HEK293T cells (1×10^6) were co-transfected with 2 μg lentiviral vector and 2 μg pPACKH1 Lentivector Packaging Kit (System Biosciences, Palo Alto, CA). The crude viral supernatant was collected 48 h and 72 h after transfection (collected viral medium, 10 mL). HOSE cells were infected with 500 μL of the collected crude viral medium per dish. The medium with infected cells was replaced with fresh medium after 24 h. During this process, the infected cells did not have with a selectable marker, and single colonies were not selected.

Short tandem repeat (STR) profiling and mycoplasma contamination test

Genomic DNA of IHOSE cells were extracted by Total DNA Extreaction Kit. (iNtRON Biotechnology, Seoul, Republic of Korea). STR profiling analysis was conducted through the

Korea Cell Line Bank (http://cellbank.snu.ac.kr). Genomic DNA was processed for STR profiling using PCR Amplification kit (Applied Biosystems, Foster, CA) according to the manufacturer's direction. After PCR amplification, the samples were analyzed on the ABI 3530xl Genetic Analyzer (Applied Biosystems) using the GeneMapper v5.0 software (Applied Biosystems). Each sample was amplified using Mycoplasma PCR Detection kit (iNtRON Biotechnology) according to the manufacturer's suggested protocol. The PCR products were separated in 1% agarose gel at 30 V for 30 min and detected using Gel Doc XR+ imaging system (Bio-Rad Laboratories, Inc, Hercules, CA)

Ion AmpliSeq Transcriptome library Preparation

When 3 HOSE, 5 IHOSE and 2 ovarian cancer cells were 70% confluence, total RNA was extracted with TRIzol Reagent according to manufacturer's protocol (Ambion, Carlsbad, CA) and were quantified using Qubit RNA HS Assay Kit (Life Technologies, Carlsbad, CA) and calculated percentage of RNA fragments larger than 200nt using smear analysis of Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). DNA samples were quantified using Qubit dsDNA HS Assay Kit (Life Technologies). An Ion AmpliSeq Transcriptome library was constructed with the Ion Transcriptome Human Gene Expression Kit (Life Technologies) as per manufacturer's protocol. 10 ng of total RNA were reverse transcribed to make cDNA by random priming. cDNA product was amplified target genes using the Ion AmpliSeq Human Gene Expression Core Panel with the Ion AmpliSeq Library Kit Plus. After primer digestion, adapters and molecular barcodes were ligated to the amplicons followed by magnetic bead purification. This library was amplified for a total of 5 cycles and purified. Amplicon size and DNA concentration were measured using an Agilent High Sensitivity DNA Kit (Agilent Technologies) according to the manufacturer's recommendation.

Ion Proton sequencing

Sample emulsion PCR, emulsion breaking, and enrichment were performed using the Ion PI Template OT2 200 Kit v3 (Life Technologies, Part #4488318 Rev. B.0), according to the manufacturer's instructions. Multiple barcoded libraries were combined together with equal molar ratios for one Ion PI v2 chip. 2 pooled Ion AmpliSeq Exome libraries were loaded onto a single Ion PI v2 chip. 5 pooled Ion AmpliSeq Transcriptome libraries were loaded onto a single Ion PI v2 chip. Subsequent emulsion PCR and enrichment of the sequencing beads of the pooled libraries was performed using the Ion OneTouch system (Life Technologies) according to the manufacturer's protocol within about 7 hours. Finally, 520 Flows sequencing was done on the Ion PI v2 chip using Ion PI Sequencing 200 Kit v3 (Life Technologies, Part #4488315 Rev. B.0) on the Ion Proton sequencer (Life Technologies).

RNA sequencing read mapping and gene expression analysis

RNA sequencing reads were mapped to the human genome (hg19) and calculated the reads count for each gene. Finally, each gene was normalized using RPKM. And we analyzed scatter plot, heat-map and differentially expressed genes (DEGs). The heat map was drawn as log values. This whole process was analyzed using DNASTAR Lasergene 15 software. David bioinformatics database (https://david.ncifcrf.gov/) was used for Gene ontology analysis.

Real-time PCR

At 70±80% of confluence, all cells were washed with PBS and total RNA was extracted with TRIzol Reagent according to manufacturer's protocol (Ambion, Carlsbad). Total RNA (1 μg) from each sample was reverse-transcribed into cDNA using Maxima First Strand cDNA Synthesis Kit

(Thermo Scientific, Waltham, MA) according to the manufacturer's protocol. Real-time polymerase chain reaction (PCR) was performed to quantify mRNA expression using SYBR Green PCR Master Mix (Enzynomics, Daejeon, Republic of Korea) and an ABI PRISM 7300 real-time PCR system (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Relative mRNA expression was quantified using the comparative Ct (ΔCt) method and expressed as $2^{-}ΔΔ^{Ct}$, where ΔΔCt = ΔE± ΔC, ΔE = CtE target±CtE GAPDH, and ΔC = CtC target±CtC GAPDH (E = experimental result and C = controls). Each assay was done in triplicate and expressed as the mean ± standard error (SE). A series of dilutions were prepared from a stock solution of total RNA to generate a standard curve to determine reaction efficiencies. The primers for PCR were as follows: `HPS1: Forward 5′-CTCCAAAAGTGAGCCCGGAT-3′and Reverse 5′-ATGAGCCTCTGCACTTGGTC-3′, KRT222: Forward 5′-AAGGGGCC TTGAAAACTCCC-3′ and Reverse 5′-GGAGGTGGCGATAAGTTGCT-3′, PKP1: Forward 5′-AGGAGGAACTCATTGCCGAC-3′ and Reverse 5′-AGCTCAGGTTCC TCAAGCAG-3′, OR522N1: Forward 5′-ACTGCAAGGGCAACGTCATA-3′ and Reverse 5′-ATCAAAGCCCCCAATCAGCA-3′, and GAPDH: Forward 5′-GAAGGTG AAGGTCGGAGT-3′ and Reverse 5′-GAAGATGGTGATGGGATTTC-3′.`

Protein extraction and western blotting

Total cell lysates were isolated using cell lysis buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1% NP-40, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM NaF, and 1 mM sodium pyrophosphate) containing proteinase inhibitor cocktail (Roche, Nutley, NJ). Protein concentrations were determined by BCA assay (Sigma-Aldrich, St. Louis, MO). Proteins were separated by SDS-PAGE and transferred from gels to 0.2 μm nitrocellulose membranes (Pall Corporation, Washington, NY). Protein bands were visualized using western blotting luminol reagent (Santa Cruz Biotechology, Inc., Dallas, Texas) after binding with a HRP-conjugated secondary antibody. Anti-Cytokeratin 7 (sc-23879), anti-Cytokeratin 18 (sc-515852), anti-EpCAM (sc-25308), anti-α-Actinin (sc-17829), and anti-GAPDH (sc-59541) antibodies were obtained from Santa Cruz Biotechnology, while anti-E-cadherin (#14472), anti-CD133 (#5860), and anti-CD44 (#3570) antibodies were purchased from Cell Signaling Technology (Danvers, MA)

Cell proliferation assay

To directly count the number of cells, cells were seeded in a 6-well plate at a density of 4×10^4 cells per well and cultured for 8 days. Ten microliters of resuspended cells were used for obtaining cell counts every 2 days using an automated cell counter (Logos Biosystems, Inc., Republic of Korea). All experiments were performed in triplicate. The growth rate was estimated between 2 and 8 days using the formula [32] :

$$N_t = N_0 2^{ft}$$

where N_t, total number of cells; N_0, initial number of cells; f, growth rate; and t, treatment time. The growth rate values were then used to calculate the doubling time, using the formula [32] :

$$\text{Doubling time} = \frac{\ln(2)}{f}$$

where f, growth rate and ln = natural logarithm.

Soft agar assay for colony formation

Cells (1×10^4) were seeded on 0.3% agar containing 10% FBS underneath 0.6% top agar in a 12-well plate. After a 1-week incubation at 37ÊCand 5% CO_2, 1 mL of media was added to the

dishes to avoid drying out and to refill sufficient nutrients. Four weeks later, colonies were stained using a 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolum (MTT) solution.

Statistical analysis

Experimental results were statistically evaluated with two-tailed paired student's t test using Graphpad Prism 7. All tests of significance were set at $p < 0.05$.

Results

Preparation and characterization of five IHOSE cell lines

We obtained HOSE cells from four patients without cancer and used a lentivirus to overexpress HPV-E6/E7 and SV40 T antigen in these cells. One of the HOSE cell samples, HOSE-1431, was used to produce two immortalized cell lines that respectively overexpressed HPV-E6/E7 or SV40 T antigen. IHOSE cell lines were established from the other three HOSE cell samples by inducing overexpression of SV40 T antigen only, because HOSE cells expressing HPV-E6/E7 exhibited senescence. Fig 1A shows images of the cells of the five IHOSE cell lines at low or high density. In previous reports, HOSE or IHOSE cells in a monolayer culture exhibited a cobblestone appearance, depending on the conditions of the culture media [33±35], and IHOSE cells infected of hTERT exhibited the co-existence of rod and cobblestone shapes [6]. The five IHOSE exhibited this co-existence with similar a morphology to that of epithelial cells (Fig 1A). Using RT-PCR, we screened for mycoplasma contamination, and confirmed no mycoplasma DNA was present in any of the cell lines (Fig 1B). Further, RT-PCR was used to confirm the successful insertion of the SV40 T antigen and HPV-E6/E7 genes for cell immortalization. All four IHOSE cell lines immortalized by SV40 T antigen expressed the antigen, whereas HPV-E6/E7 was expressed in the other IHOSE cell line. HPV-E6/E7 protein expression was also detected from total RNA samples of SNU-17 and Caski cervical cancer cells as positive controls (Fig 1C). Western blotting was used to detect protein expression of the epithelial cell markers cytokeratin 7 and cytokeratin 18 in IHOSE cells; cytokeratin 18 showed relatively high expression in both IHOSE cells and ovarian cancer cells, whereas cytokeratin 7 was expressed at lower levels in IHOSE cells compared to that found in ovarian cancer cells. However, both cytokeratins were expressed at lower levels in cells from the IHOSE-0160-SV40 cell line and SKOV3 ovarian cancer cells than in the other cells tested (Fig 1D). This difference is consistent with the well-known low expression of cytokeratin genes in SKOV3 cells [36, 37]. From RNA sequencing analysis, we determined that the major expressed cytokeratins in IHOSE and ovarian cancer cells were keratin types 7, 8, 18, and 19. The expression of these cytokeratins in IHOSE cells decreased after immortalization. Especially, the expression level of these keratins was diminished more in IHOSE-0160-SV40 cells than in the other IHOSE cell lines (S1 Table). For the five IHOSE and two ovarian cancer cell lines, the growth rates were measured for up to 8 days. The growth rates of five IHOSE cells differed from each other. 8695-SV40 and 1431-SV40 grew faster than the other IHOSE cells, and 1431 E6/E7 grew the slowest. Compared to the ovarian cancer cell lines, 8695-SV40 and 1431-SV40 grew faster than SKOV3, but their growth was similar with OVCAR3 (Fig 1E and Table 1). Table 1 indicated the doubling time of each cell lines. Genomic DNA was extracted from cells from all five IHOSE cell lines, and DNA fingerprinting was conducted using 16 STR loci. Comparison of STR profiles with the integrated molecular authentication database 2.1 [38] confirmed that all five IHOSE cell lines represent novel cell lines (Table 2). As IHOSE-1431-E6/E7 and IHOSE-1431-SV40 cells share the common HOSE progenitor cell HOSE-

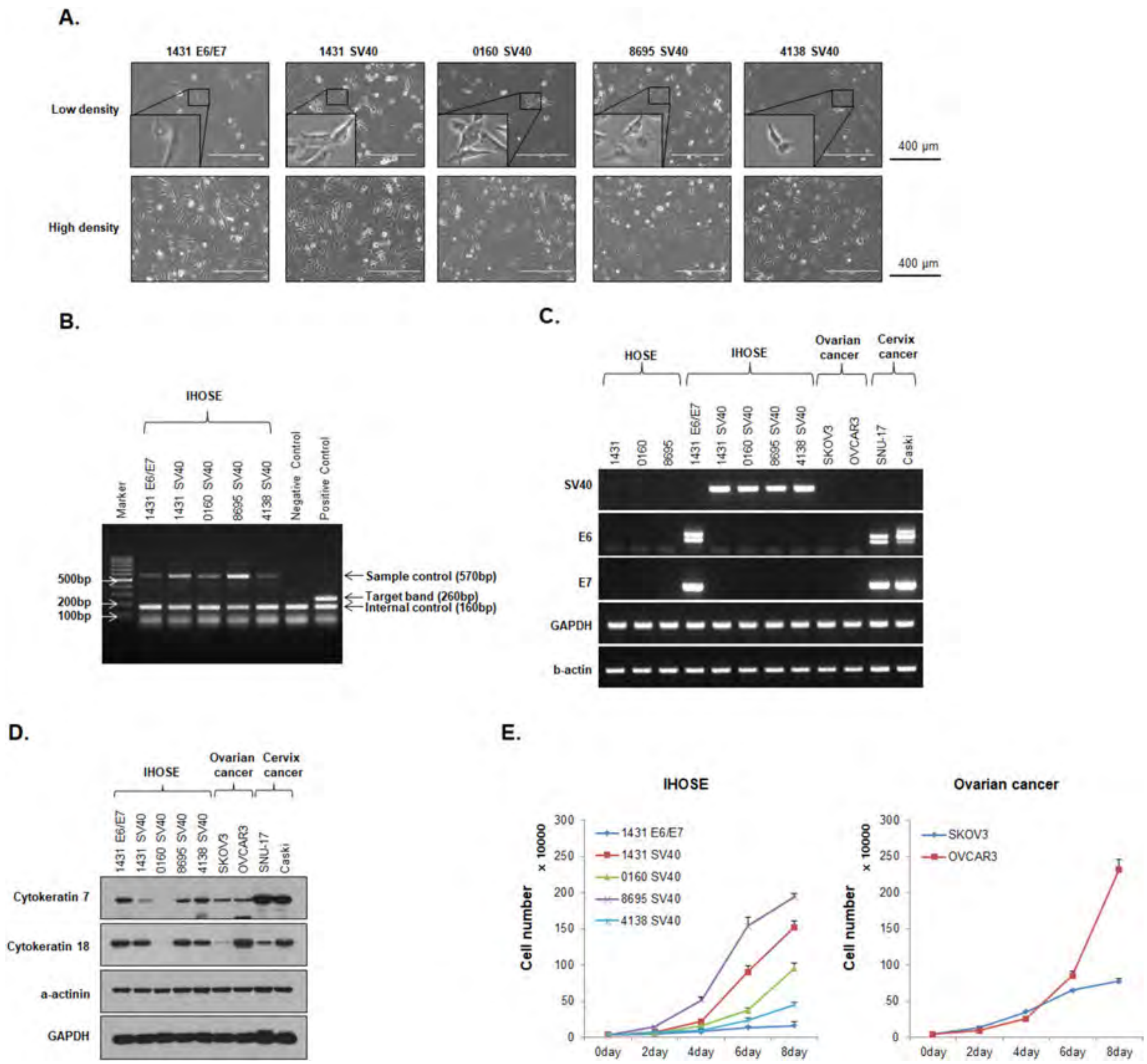

Fig 1. Characterization of five immortalized human ovary surface epithelial (IHOSE) cell lines. (A) Representative images showing the morphology of cells from five IHOSE cell lines. SE (Upper panel = Low density of cells; Bottom panel = High density of cells). Scale bar: 400 μm, 100× magnification. **(B)** Mycoplasm contamination testing was performed using 100 ng genomic DNA by RT PCR. **(C)** The expression levels of SV40 T antigen and HPV E6/E7 protein in IHOSE cells were detected by RT PCR. β actin and GAPDH were used as loading controls. **(D)** The expression levels of indicated protein were determined by western blot analysis in the five IHOSE and cancer cell lines. β actin and GAPDH were used as loading controls. **(E)** Cells (4×10^4) were seeded in a 6 well plate and counted every 2 days up to 8 days. Cell proliferation was determined using an automated cell counter. Results indicate cell number ± SD.

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1431, identical STR profiling results were obtained for these two cell lines. Consequently, we have established five IHOSE cell lines capable of continuous growth.

IHOSE cells are genetically closer to HOSE cells than ovarian cancer cells

RNA sequencing was then performed to examine the gene expression patterns in the newly established IHOSE cells in comparison to three of the progenitor HOSE cell lines and two ovarian cancer cell lines. Based on expression data of 22,878 genes (S3 Table), correlation (R^2) values among the three groups were analyzed through comparison of scatter plots between groups. The correlation in gene expression levels was greater between the IHOSE and HOSE

Table 1. The doubling time of each cell lines.

Cell lines	Doubling Time (h)[a]	Std. Deviation
1431 E6/E7	90.20	11.52
1431 SV40	35.81	1.55
0160 SV40	42.92	1.17
8695 SV40	33.67	2.21
4138 SV40	52.11	2.47
SKOV3	46.02	1.12
OVCAR3	33.98	0.67

The doubling time was calculated using results between 2 and 8 days in Fig 1E.

[a](h) = hour

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groups (R^2 = 0.9288) than that found between IHOSE or HOSE and cancer cells (R^2 = 0.8562 and R^2 = 0.7982, respectively) (Fig 2A). Gene expression patterns of the 319 differentially expressed genes (DEGs) were obtained based on a signal threshold ≥ 0.1, $p \leq 0.05$, false discovery rate of 5%, and two-fold alteration. There were 37 DEGs identified between the IHOSE and HOSE groups, 278 DEGs identified between the IHOSE and cancer groups, and four DEGs among all three groups (Fig 2B and S2 Table). The heatmap representing the expression patterns of these 319 DEGs revealed little difference in gene expression between the HOSE and IHOSE groups, with significant differences evident for the cancer group. This region of significant difference in gene expression was categorized into gene cluster 1 and gene cluster 2 (Fig 2C), reflecting 102 and 67 genes with reduced and enhanced expression in the ovarian cancer group, respectively (Fig 2D). Gene Ontology (GO) analysis of these clusters showed that gene cluster 1 could be divided into 25 GO terms, with "mesenchyme migration" and "cell adhesion" included among the top eight terms. Gene cluster 2 was divided into eight GO terms, with "detection of chemical stimulus involved sensory perception of smell" and "G-protein coupled receptor signaling pathway" ranked highest (Fig 2E). In addition, the dendrogram constructed for each cell type according to the gene expression pattern revealed that IHOSE cells clustered closer to HOSE cells, which were clearly distinguishable from ovarian cancer cells (Fig 2F). To

Table 2. STR profiling of five immortalized human ovary surface epithelial cells.

STR Loci	1431 E6/E7	1431 SV40	0160 Myc/SV40	8695 SV40	4138 SV40
D8S1179	14, 17	14,17	12, 13	13, 14	10, 16
D21S11	29, 33.2	29, 33.2	30	29, 33.2	30
D7S820	10, 13	10, 13	12	12	11
CSF1PO	10, 12	10, 12	11, 12	9, 10	12, 13
D3S1358	15	15	15,18	15, 17	16, 7
TH01	7, 9	7, 9	6, 9	9	7, 9
D13S317	8, 12	8, 12	10	8, 10	10, 12
D16S539	9, 11	9, 11	10, 12	9, 11	9, 13
D2S1338	19, 20	19, 20	23, 24	18, 25	23, 24
D19S433	13, 14	13, 14	13, 14.2	13, 14.2	13, 14
Vwa	18	18	18, 19	16	17, 19
TPOX	8, 11	8, 11	8, 9	8	9, 11
D18S51	15, 16	15, 16	13, 14	13, 17	13, 14
Amelogenin	X	X	X	X	X
D5S818	11, 12	11, 12	10, 12	11, 12	10
FGA	24, 25	24, 25	21, 23	10, 12	26

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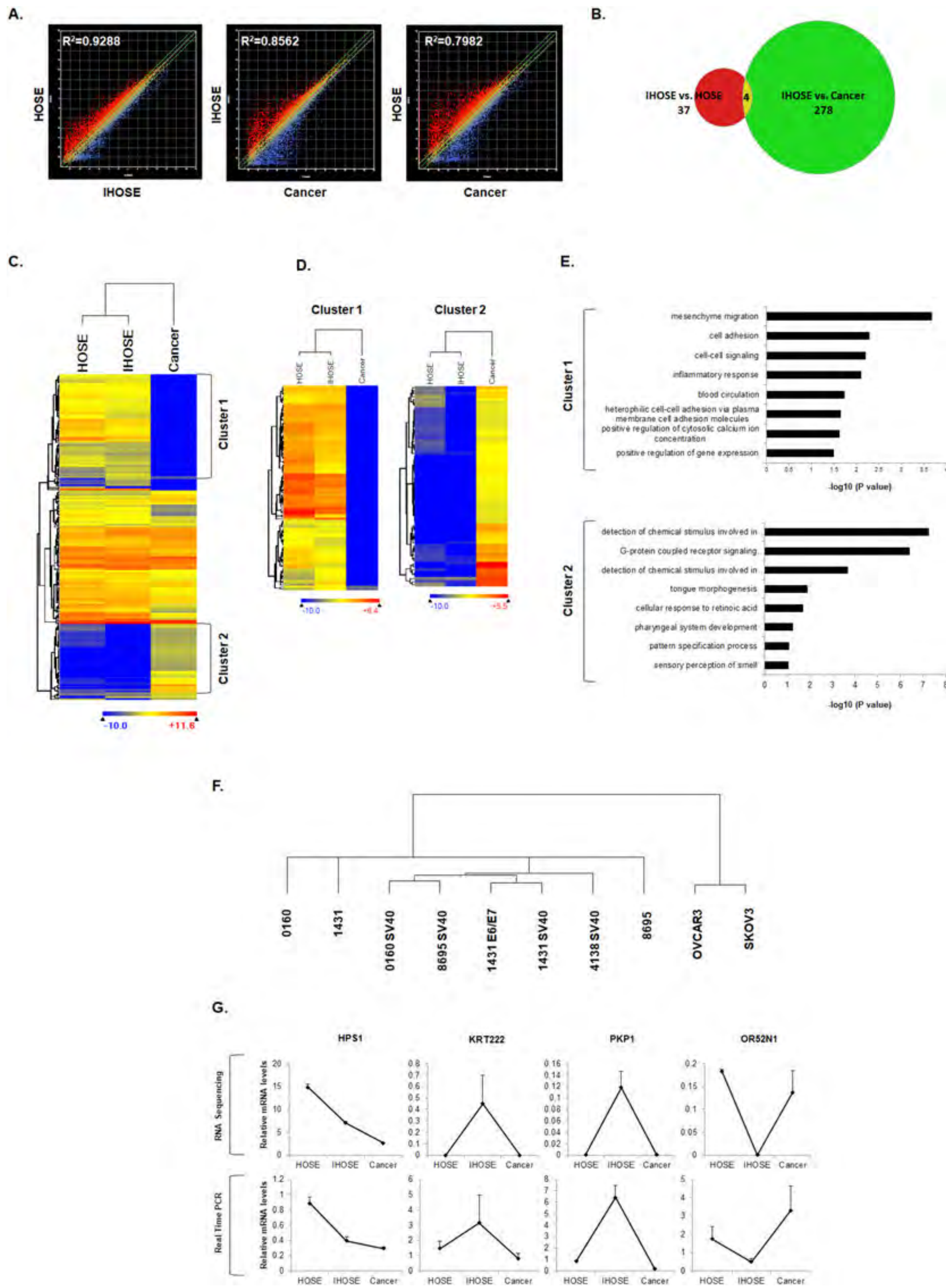

Fig 2. Analysis of differentially expressed genes (DEGs) between IHOSE, HOSE, and ovarian cancer (Cancer) cells using RNA sequencing. **(A)** Scatter plot revealing a linear correlation of gene expression pattern according to comparative analysis in HOSE versus IHOSE, IHOSE versus Cancer, and HOSE versus Cancer. **(B)** Venn diagram showing a common denominator in 319 DEGs based on sorting conditions of 2 fold change, a read count threshold > 0.1, and p < 0.05. **(C)** Heatmap of 319 DEGs in three analyzed groups. **(D)** Heat map of downregulated genes (Cluster 1) or upregulated genes (Cluster 2) in ovarian cancer compared to HOSE and IHOSE. **(E)** Gene ontology related to biological process ordered according to p value (log10). Functional annotation clustering was performed using the DAVID algorithm (Upper panel = Cluster 1; Lower panel = Cluster 2). **(F)** Dendrogram for the clustering of each cell with Euclidean distance metric. **(G)** Validation of RNA sequencing gene expression levels by real time PCR for four common DEGs in the three groups. Data are represented as the mean fold change ± SE (Upper panel = RNA sequencing; Lower panel = Real time PCR).

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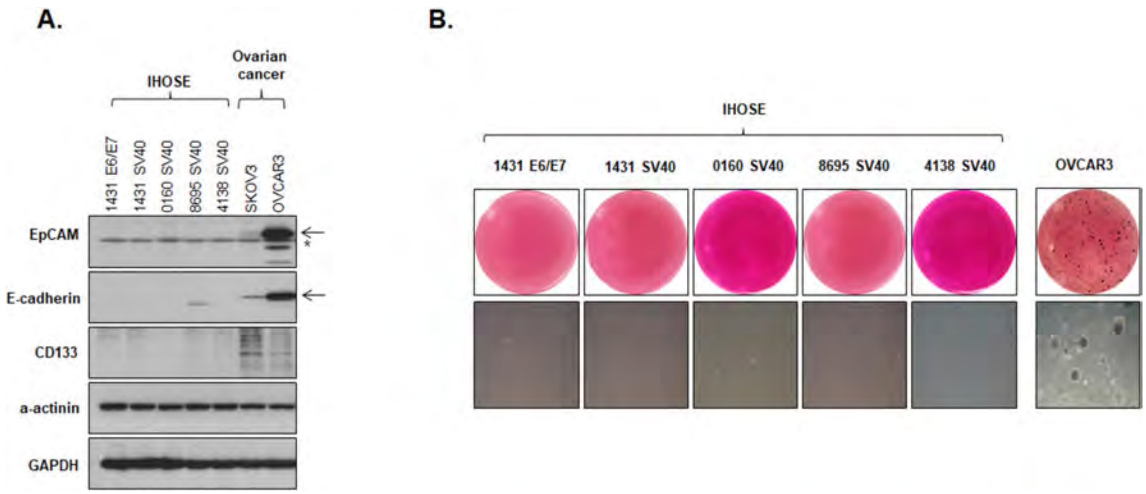

Fig 3. Immortalized human ovary surface epithelial (IHOSE) cell lines have no malignant characteristics. (A) The expression levels of indicated protein for the cancer stem markers EpCAM, CD133, and E cadherin were measured by western blot analysis (Star = non specific band). **(B)** Soft agar colony formation of 5 IHOSE and OVCAR3 cell lines stained by 5 mg/mL MTT solution. Representative images of wells and colonies (Colonies = 100× magnification).

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verify the accuracy of the RNA sequencing results, real-time PCR was performed for the four commonly altered genes found in the three groups (Fig 2B). Despite slight differences in the degree of variation among groups, the patterns of increased or decreased gene expression among groups were consistent to those found from RNA sequencing (Fig 2G, top) and real-time PCR results (Fig 2G, bottom). Overall, these findings demonstrate that IHOSE cells have a more similar gene expression pattern to HOSE cells than to ovarian cancer cells, indicating their suitability as control cell lines for cancer research.

IHOSE cells have no malignant features of cancer cells

IHOSE cell lines were established by overexpressing immortalizing proteins to maintain continuous cell division. Since continuous cell division is a prominent feature of cancer cells, we verified whether these IHOSE cells have the malignant features of cancer cells by detecting protein expression levels of cancer stem cell markers using western blotting. We found that two cancer stem cell markers, EpCAM and CD133, were expressed only in ovarian cancer cells, and the expression level of the surface marker E-cadherin was much higher in ovarian cancer cells than in cells from any of the five IHOSE cell lines (Fig 3A). Another feature of cancer cells is able to growth in suspended state. Using the anchorage-independent soft agar assay, we demonstrated that the IHOSE cells did not form colonies as opposed to the ovarian cancer cells that formed colonies (Fig 3B). Therefore, based on the previous results of the RNA sequencing analysis, we showed that the IHOSE cells are more similar to the HOSE cells than the ovarian cancer cell lines. Moreover, the IHOSE cells exhibited low expression of cancer stem cell markers and lack of anchorage-independent growth. Hence, we concluded that the IHOSE cells did not show carcinogenic characteristics of ovarian cancer cells, but they still exhibited the desired property of continuous cell division.

Discussion

We have been exploring differences in gene expression between the HOSE and ovarian cancer cell lines for more than a decade and have been identifying the clinical implications of various

ovarian cancer markers [39±43]. However, culturing of HOSE cells has proven to be a challenge; moreover, the diversity of HOSE pool doesn't appear to be sufficiently secure. This study was initiated as part of our efforts to directly secure IHOSE cells and to have diversity in the experimental control group, while recognizing that immortalization of HOSE cells, which has previously been conducted in various laboratories, may be one method. Additionally, several papers related to the origin of ovarian cancer have been published in recently. It has been reported that epithelial ovarian cancer cells originate from other organs, and not from the ovaries. Serous tumors have been reported to occur in the epithelial cells of the fallopian tube, and endometrioid and clear cell tumors have been reported to be associated with endometriosis which generally occurs in endometrial tissue due to retrograde menstruation. Thus, the endometrium is also a origin site for these ovarian tumors [29, 31, 44]. Ovarian cancer is a heterogeneous disease that consists of several types of tumors with very different clinicopathological features and behaviors. Therefore, the origin of ovarian cancer is likely to vary. In order to study ovarian cancer, it is necessary to secure normal cells from other pelvic organs in addition to the normal ovarian epithelial cells. Our future goal is to obtain a variety of normal cells that are associated with the development of ovarian cancer, in order to produce a cell line and use it to study ovarian cancer. By the way, a suitable source of carcinogenic tissue and cell lines is essential for research on ovarian cancer to identify candidate biomarkers. However, such studies have been hindered owing to the challenge of obtaining histologically stable samples of HOSE cells as controls. Moreover, even if HOSE cells can be obtained, their use is limited to single short-term experiments because of aging-induced damage that prevents continuous cell division. Thus, the acquisition of HOSE cells and establishment of immortalized cell lines are essential preparative steps to advance the field of ovarian cancer research. Recently, the development of advanced next-generation sequencing technology enables detailed genomic analysis of tumor and cell lines as well as investigation into the key genes and molecular events involved in carcinogenesis [45]. Among these techniques, RNA sequencing is a powerful method for accurately detecting over 10,000 changes at the RNA level using only a small amount of RNA. Thus, we employed an RNA sequencing strategy to compare gene expression profiles of the newly established IHOSE cell lines with each of their progenitor HOSE cells and ovarian cancer cells, demonstrating that IHOSE cells had retained most of the gene expression profiles of their progenitor cells, and were distinct from cancer cells. Notably, the two types of IHOSE cells (1431-E6/E7 and 1431-SV40) established by overexpressing HPV-E6/E7 and SV40 T antigen from the common progenitor HOSE-1431 exhibited identical STR profiles. However, the cell growth rates of these two IHOSE cell lines differed by over two-fold, indicating a strong influence of the immortalization on cell growth properties. Nevertheless, the short Euclidean distance between IHOSE-1431-E6/E7 and IHOSE-1431-SV40 on the dendrogram confirmed that these differences in growth rates were not due to alterations in gene expression during the immortalization process. Further, cells from these two IHOSE cell lines do not seem to have significant differences in overall gene expression compared to cells from the other IHOSE cell lines or progenitor HOSE cells. The consistency between HOSE and IHOSE cells was further confirmed through analysis of DEGs, with clear differences detected from the gene expression patterns of ovarian cancer cells. The gene cluster showing enhanced expression in the cancer group was enriched in the GO term `G-proteincoupled receptor signaling pathway', a finding which is consistent with a previous study showing a close association of G-protein-coupled receptors with the development, progression, and metastasis of ovarian cancer [46]. In general, when cell adhesion decreases, cell growth increases along with an increase in migration or invasion [47, 48]. However, the RNA sequencing and GO results are in conflict with this general pattern, demonstrating reduced expression levels of genes involved in cell adhesion and mesenchyme migration in ovarian cancer cells. Cell adhesion molecules are

widely known as tumor suppressors because they promote cell adhesion-mediated contact inhibition to reduce cell growth and inhibit tumor dissemination [48]. However, there are also some cell adhesion molecules that can promote cell migration or invasiveness. For example, increasing the expression level of hepaCAM in HepG2 and MCF7 cells results in enhanced cell-extracellular matrix adhesion and cell migration [49]. In addition, overexpression of CEACAM1 in thyroid cancer cells results in increased cell invasion and migration, while CEACAM1 knockdown improved cell growth but decreased cell invasiveness [50]. Based on this background, the present results may imply reduced expression of genes governing migration along with reduced expression of certain cell adhesion molecules in ovarian cancer cells. We further detected a clear difference in the expression of cancer stem cell markers and tumor formation ability between IHOSE cells and ovarian cancer cells. Unfortunately, we were not able to conduct this comparison with additional progenitor HOSE cells because of the limited quantity of cells. We also found that the cancer stem cell markers EpCAM, CD133, and E-cadherin were either expressed at low levels or were absent in IHOSE cells, and none of the five IHOSE cell lines formed colonies in the anchorage-independent soft agar assay. Thus, despite exhibiting immortalization characteristics for the continuous growth of IHOSE cells, these cells do not show the genetic or phenotypic characteristics of cancer cells. Therefore, the established IHOSE cells should be highly useful as a control cell source for ovarian cancer studies, and thus, are expected to become indispensable in basic research on ovarian cancer.

Supporting information

S1 Table. Cytokeratin expression in RNA sequencing data.
(XLSX)

S2 Table. Gene lists and RNA-sequencing read counts in venn diagram.
(XLSX)

S3 Table. Whole gene list and read counts (22,878 genes).
(XLSX)

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Writing ± review & editing: Ha-Yeon Shin, Wookyeom Yang, Jae-hoon Kim.

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Exhibit 100

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Review Section

BIOLOGICAL EFFECTS OF COSMETIC TALC

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Summary—A review of the literature reveals two primary issues: (1) a weak, but not causal, association of hygienic use of cosmetic talc and ovarian cancer; (2) lung changes in animals exposed to talc aerosol concentrations that resulted in lung overload. The evidentiary weight of the most significant of the epidemiological and laboratory studies and their biological significance for human risk assessment are briefly discussed. Publications describing granulomatous lesions attributed to talc on surgical gloves, and consequences of accidental inhalation of baby powder by infants are also reviewed. The literature reviewed does not provide any convincing evidence that pure cosmetic talc, when used as intended, presents a health risk to the human consumer.

Introduction

Hildick-Smith (1976 and 1977) and Lord (1978) have reviewed the older literature relating to talc. Hildick-Smith concluded that the normal use of cosmetic-grade talc does not present a health hazard. In his review of biological effects of talc in laboratory animals, Lord found the mineral to be fibrogenic but he observed that the fibrotic response was a function of the administered dose and 'that there are levels of exposure that are tolerable'. In none of the studies reviewed was there any indication of neoplasia.

A computer search of the literature of the last 15 years provided well over 700 titles/abstracts on various aspects of talc and talc issues. Of these, 137 were selected for review as they describe epidemiological, clinical and relevant animal and *in vitro* studies. Papers on exposures to *industrial* talc, often containing more toxic impurities, such as asbestos fibres, were excluded as irrelevant to the safety of *cosmetic* talc when used as intended. Studies of lesions following intravenous injection of talc by drug addicts were also excluded because of unintended use. The term 'cosmetic talc' in this review refers to talc of high purity as produced by today's responsible manufacturers.

A review of the selected titles reveals two primary issues, namely (1) hygienic use of cosmetic talc and ovarian cancer risk, and (2) health effects of inhaled cosmetic talc. In addition, a number of papers describe granulomatous lesions attributed to talc on surgical gloves, and consequences of accidental inhalation of baby powder by infants. This review focuses on publications dealing with these and related issues.

Hygienic use of talc and ovarian cancer risk

The Harlow paper

Publication of a paper by Harlow *et al.* (1992) revived concern that chronic talc use in genital hygiene might be associated with an increased risk of epithelial ovarian cancer. That concern was originally raised 21 years earlier in a paper by Henderson *et al.* (1971) on the same subject. Harlow *et al.* (1992) provided the most detailed results to date on the relationship between perineal talc exposure and ovarian cancer. The study is the only one to date designed specifically to address this issue. It is among the largest studies (greatest statistical power) and one of the few to use closely age-matched neighbourhood controls.

The authors interviewed 235 white women diagnosed with epithelial ovarian cancer between 1984 and 1987 in Boston and 239 population-based matched controls. Approximately 50% of cases and 40% of controls used talc on the perineum, undergarments, sanitary napkins or diaphragms. This resulted in a 1.5 odds ratio (OR) for ovarian cancer [95% confidence interval (CI) 1.0–2.1]. Perineal exposure to talc resulted in significantly elevated risks in the subgroups who applied it directly as a body powder (OR 1.7, 95% CI 1.1–2.7), on a daily basis (OR 1.8, 95% CI 1.1–3.0), and for more than 10 years (OR 1.6, 95% CI 1.0–2.7). The greatest risk was

Abbreviations: BAL = bronchoalveolar lavage fluid; CI = confidence interval; NTP = National Toxicology Program; OR = odds ratio; RR = relative risk; SCE = sister chromatid exchange; UDS = unscheduled DNA synthesis.

observed in women with more than 10,000 talc applications during years when they were ovulating and had an intact genital tract (OR 2.8, 95% CI 1.4–5.4); however, this applied to only 14% of the women with ovarian cancer.

The authors acknowledge the inherent limitations of epidemiological studies. In their statistical analyses they adjusted for several confounding variables and conceded the existence of additional but intangible variables. In-person interviews included enquiries into dietary history but it is not clear whether, for example, lactose consumption and coffee consumption were determined and adjusted for; both substances have been associated with a significant increase in the risk of ovarian cancer (Cramer *et al.*, 1989; Whittemore *et al.*, 1988). Except for these two cases, no diligent efforts appear to have been made to identify other confounding variables that could account for increased incidence of ovarian cancer, such as vulvovaginal diseases (e.g. yeast infections and trichomoniasis) and obesity. McGowan *et al.* (1979) reported a higher incidence of rubella infection between the ages of 12 and 18 years in ovarian cancer patients than in controls, but there was a lower frequency of mumps (West, 1966) and of pneumonia and influenza (Joly *et al.*, 1974).

Harlow *et al.* (1992) mention in one sentence that they used meta-analysis in the statistical evaluation of their epidemiological data. Meta-analysis requires rigid criteria that must be strictly observed to yield meaningful results (Chalmers *et al.*, 1989; Yusuf *et al.*, 1985). Without details on the meta-analytical procedures used by the authors it is not possible to determine whether the use of meta-analysis was appropriate, and if so, whether it was used appropriately.

The sample population in the Harlow study is limited in numbers and restricted to white women in the Boston area. With an overall adjusted OR of 1.5 (CI 1.0–2.1) the results are statistically barely significant. Extrapolating from results in that small, restrictive group to the general population will require caution.

The authors conclude that their "data support the concept that a life-time pattern of perineal talc use may increase the risk for epithelial ovarian cancers", but they do not claim to have established a cause–effect relationship.

Related literature

Harlow *et al.* (1992) cite a number of literature references on talc translocation and putative association between hygienic use of talc and ovarian cancer. These references contribute to the ambiguity of the database as some of them suggest translocation or support an association whereas others do not; again, others suggest alternative explanations for greater-than-average incidences of ovarian cancer. For example, Cramer *et al.* (1989) suggested that lactose consumption might be a dietary risk factor and transferase a genetic risk factor. Whittemore *et al.* (1988) observed in a well-designed study (188 cases, 539 controls) that the ovarian cancer risk in women who had consumed coffee for more than 40 years was 3.4 times that of women who had never regularly consumed coffee. This relationship is more robust ($P = 0.01$) than the marginal significance of the relationship with perineal talc use [relative risk (RR) = 1.40, $P = 0.06$].

A causal relationship between talc and ovarian cancer requires that talc particles, administered to the perineum or to the vagina, can translocate to the ovaries. Whether or not inanimate particles without locomotion of their own can do this without assistance has been the subject of a number of investigations in humans and animals. Results have been inconsistent and ambiguous. For a discussion of relevant findings published before 1986, reference is made to Wehner *et al.* (1986).

Henderson *et al.* (1986) observed talc particles in the ovaries of all eight ex-breeder rats after *intrauterine* deposition of a talc suspension. Following *intravaginal* deposition of talc suspension, talc particles were found in the ovaries of only two of six ex-breeder rats. Retrograde flow of menstrual products into the peritoneal cavity through the oviducts is well known; therefore, translocation to the ovaries of talc particles deposited in the uterus in 250 μl saline, perhaps aided by the hydrostatic pressure of the saline solution during and after deposition, is not surprising. Yet in two of six rats the inanimate talc particles managed to cross the cervical barrier and reach the ovaries, perhaps also aided by hydrostatic pressure during deposition. Henderson *et al.* (1986) express a guarded opinion on the association of talc with cancer, stating that "carcinogenic activity of talc has not been established although its ubiquitous presence in the environment and its elemental similarity to asbestos has brought it under suspicion". Elemental similarity, of course, does not necessarily mean similarity in biological effects. It is well known that differences in physical properties, such as shape and surface characteristics, between elementally similar substances are responsible for significant differences in their biological effects. The pharmacological action of certain elementally and structurally identical agents (isomers) depends on whether they are dextrorotatory or levorotatory.

A remarkably large number of talc particles were found by Henderson *et al.* (1979) in human ovarian tissue, namely from 6900 to 55,100 particles/g wet weight tissue in three normal ovaries, from 17,400 to 24,300 in three cystic ovaries, and from 6400 to 24,300 in three ovarian adenocarcinomas. In another reference (Henderson *et al.*, 1978), 2×10^5 particles/mm^3 are reported from oxygen incineration studies of human ovarian tissue.

Talc particles in human and animal tissues can be identified by X-ray fluorescence and X-ray diffraction

(Wehner *et al.*, 1977c) and by neutron activation of talc before animal exposure and subsequent γ-ray analysis of animal tissues for at least two suitable radionuclides (Wehner *et al.*, 1986 and 1977b). The neutron activation technique eliminates contamination problems during sample collection and processing.

Talc particles found, for example, *on* ovarian tissue might be contaminants deposited during sample collection and processing. For talc particles *in* ovarian tissue, contamination during sample collection and processing can be ruled out.

Wehner *et al.* (1985 and 1986) investigated talc translocation in Cynomolgus monkeys (*Macaca fascicularis*). The female of this species resembles the human female closer than any other animal model in the physiological and anatomical parameters of interest. The authors deposited a suspension of neutron-activated talc in the posterior fornix of the vagina on 30 consecutive work days (45 calendar days), that is, through at least one menstrual cycle (Wehner *et al.*, 1986). 2 days after the 30th talc application the animals were killed. The following samples underwent γ-ray analysis: vagina with the cervix of the uterus; uterus; the entire oviduct in three sections; ovaries; and peritoneal lavage fluid. Only the vagina-with-cervix samples—the site of deposition—contained varying quantities of talc. No talc was detected in any of the other samples. The detection limit of the neutron activation/γ-ray analysis technique under the described experimental conditions was approximately 0.5 μg talc (Wehner *et al.*, 1986) or about 1/230,000–1/245,000 of the estimated talc deposition in the posterior vaginal fornix (Wehner *et al.*, 1985).

Harlow and Weiss (1989) interviewed 116 patients with borderline ovarian tumours on their use of hygienic powders and found 'no appreciably altered risk' in the use of baby powder or cornstarch. However, the unspecified smaller number among those women who use deodorizing powders alone or in combination with other talc-containing powders had a 2.8-fold higher risk than 158 women without perineal exposure to powder. In the light of this newly discovered confounding variable, Harlow and Weiss recommend that the specific type(s) of powder used should be identified in future studies on hygienic powder use and ovarian tumours.

Cramer *et al.* (1982) observed a statistically significant ($P < 0.003$) relationship between epithelial ovarian cancer and talc used for dusting the perineum or sanitary napkins in 215 women. However, Cramer *et al.* (1982) found no relationship between ovarian cancer and talc exposure from dusting condoms or diaphragms, even though talc, in the latter applications, is deposited close to the cervical os. Hartge *et al.* (1983) made a similar observation from their epidemiological study. Their data indicate that the use of talc on a diaphragm did not appear to increase risk and that there was no overall association between talc use and risk of ovarian cancer although a small group of women who specifically reported 'genital use' of talc showed an unspecified excess relative risk.

Mostafa *et al.* (1985) observed in 175 grossly normal, surgically removed human ovaries a 9% incidence of magnesium silicate granulomas and an additional 9% incidence of histological changes very similar to these granulomas.

Booth *et al.* (1989) reported an association between infertility as well as late onset of menopause and increased risk of ovarian cancer. In their opinion, the evidence linking talc with ovarian cancer was controversial, and they stated that more studies are needed to clarify this issue.

The association between 'fibre' exposure and ovarian cancer has been described by Rosenblatt *et al.* (1992). Cases were ascertained between 1981 and 1985. The authors define fibre exposure "as exposure to asbestos, talc (which may contain asbestos), and fiberglass". The authors observed elevated, but statistically not significant, risks with the use of condoms, powdered diaphragms and genital bath-talc. The risk for use of talc on sanitary napkins was significantly greater than unity (RR = 4.8; 95% CI = 1.3–18.0). The authors conclude that "The results of our study and others suggest that genital fiber exposure may be associated with an adverse effect ... but further study is needed to determine if this relationship is causal in nature."

Tzonou *et al.* (1993) examined the use of analgesics, tranquillizers and perineal talc application as risk factors for ovarian cancer in 189 cases and 200 controls. Talc use was determined on a no/yes basis without attempts to quantify application. The authors adjusted for a number of confounding variables, among them age, years of schooling, body weight, age at menarche, menopausal status, parity (nulliparous/parous, parous age at first birth, smoking (non-smoker/ever smoker), consumption of alcohol (glasses/day) and coffee (cups/day). There was a marginally significant inverse association with an apparent dose–response trend between frequency of analgesics use and ovarian cancer, and a highly significant dose-dependent positive relation between hair dyeing and ovarian cancer. As to talc, the authors state, "although the number of talc users is in general small and the respective confidence interval fairly large, there is clearly no evidence of an increased risk associated with perineal application of talc."

Chen *et al.* (1992) investigated risk factors for epithelial ovarian cancer in Beijing in 112 cases and 224 age-matched community controls. Among a number of other risk factors, the authors reported in their abstract an elevated risk in women with a history of long-term (>3 months) application of talc-containing dusting powder to the lower abdomen and perineum (RR 3.9. 95% CI 0.9–10.63).

Examination of the full paper reveals that these figures are based on seven (!) cases and five (!) controls.

Kupryjanczyk (1989) described multiple talc granulomas, inclusion cysts and adhesions in close proximity to an adenomatoid tumour of the left ovary of a 41-year-old patient. She suggests that talc crystals, as well as repair and inflammatory processes, should be taken into account as initiating factors in the development of ovarian adenomatoid tumours in susceptible patients.

In their paper on the aetiology of ovarian cancer, Baylis *et al.* (1986) conclude, "It certainly cannot be said at present that talc causes ovarian cancer." However, because the cause of ovarian cancer is unknown, and because of the "unexpected and unwarranted" presence of talc in ovarian cancer tissue and talc's "chemical similarity with asbestos", the authors are pursuing further investigations.

In their review paper on the epidemiology of ovarian cancer, Greene *et al.* (1984) summarize the role of talc and asbestos as follows: "while the above observations [reviewer's comment: referring to some of the publications reviewed here] are provocative, a conclusive role for talc or asbestos or both in the genesis of human ovarian cancer has yet to be demonstrated in either cohort or case–control studies."

There are other papers in which an association between hygienic talc application and ovarian cancer is mentioned. Most are reviews, referring to the literature reviewed here. None provide new information on the talc/ovarian cancer issue.

Hamilton *et al.* (1984) injected 100 μl saline containing 10 mg talc into ovaries of rats and observed no evidence of neoplasia.

In their review, Longo and Young (1979) point out that "epidemiological data could be interpreted as showing that the risk of developing cancer from an occupational talc exposure was due to contaminating asbestos." This could equally apply to chronic exposure from hygienic use of talc. The authors support this view by subsequently stating "... consumer talc products marketed before 1973 were variably contaminated by asbestos", and, later, "... data collected on populations exposed before 1976 may reflect the hazard of contaminating asbestos rather than talc. Unfortunately, adherence to the revised Cosmetic, Toiletry and Fragrance Association guidelines is voluntary ... so that, even now [1979; year added], commercial talcs are not certain to be asbestos-free." Cralley *et al.* (1968) found fibre contents ranging from 8 to 30% in cosmetic talc products available at the time of their investigation.

In considering mechanisms that might be involved in ovarian carcinogenesis, Venter (1981) points to the extremely high concentrations of gonadotrophins and potent steroids in the follicular fluid that is released monthly into the pelvic cavity by the rupture of the ovarian follicles. The concentration of these chemicals correlates with the mitotic and biosynthetic activities of granulosa cells, of which approximately 50 million accumulate in a follicle during the follicular phase with about 6.5 ml of antral fluid before the follicle is transformed into a corpus luteum. Given the fact that oestrogens cause proliferation of certain cells, Venter proposes the hypothesis that the antral fluid could act as an ovarian cancer promoter. Dietl *et al.* (1986) emphasize that the ovarian surface epithelium is a dynamic tissue with distinct morphological differentiations: it may proliferate inwards and form crypts and inclusion cysts or it may develop superficial papillary excrescences. In addition, constant metaplastic changes may take place in various parts of the müllerian epithelium. These growth processes appear to be influenced by endogenous and exogenous factors. It is conceivable that these factors, in combination with the physiological, biochemical and morphological characteristics of the ovarian tissue, can induce neoplastic lesions in the ovarian surface epithelium. The repeated breaks in the epithelium that occur during ovulation apparently increase the risk of developing neoplasia.

Biological effects of inhaled talc

The literature search revealed that publications on biological effects of inhaled cosmetic talc are sparse and basically fall into two categories: (1) findings in animal studies; (2) accidental inhalation of large quantities of talc by infants and small children.

Talc pneumoconioses in adults

Feigin (1989) distinguished between three forms of pulmonary disease caused by talc inhalation, namely (1) talcosilicosis, (2) talcoasbestosis and (3) pure talcosis. Feigin writes: "Talcosilicosis is produced by exposure to talc usually from Italy but also from California associated with silica and other non-asbestiform minerals.... The clinical and radiographic manifestations resemble those of silicosis.... The only documented difference between silicosis and talcosilicosis is the presence of talc, as demonstrated histologically. No other differences have been described. The radiographic changes are indistinguishable from those of silicosis." Talcoasbestosis is caused by inhalation of talc containing asbestiform fibres, such as tremolite and anthophyllite, as mined, for example, in upper New York state. On pure talcosis, Feigin writes: "Symptoms and pulmonary function test results consistent with restrictive pulmonary disease are well documented in pure talcosis; airway obstruction may also occur. The clinical form of the disease has most often been documented in miners and other workers involved in the obtaining and processing of pure talc. It has also been documented in people exposed to cosmetics, but only when the exposure was very heavy and prolonged".

This reviewer could find only two literature references on talcosis in consumers. Wells *et al.* (1979)

describe a case of talcosis due to talc abuse, initially suspected to be tuberculosis, in a 41-year-old housewife. On direct questioning the patient admitted to very heavy (at least once a day) use of talc powder over her whole body in an unventilated room for many years. The other reference is to a paper by Tukiainen *et al.* (1984) who describe two cases. One of them involved an elderly female smoker of 20 years with a history of several operations. When she presented years later with non-productive cough, dyspnoea, tachycardia and low-grade fever, talcosis was considered a possibility on account of her 10-year use of talcum powder two or three times a day in an unventilated room. The authors eventually diagnosed chronic sarcoidosis with talc deposition in the lungs. The other case was an elderly female smoker of 30 years who had been occupationally exposed to *industrial* talc from 1958 to 1968.

Scatarige and Stitik (1988) reviewed the literature on the induction of thoracic malignancies in inorganic dust pneumoconiosis. They concluded that "talc pneumoconiosis does not appear to be associated with an increased risk of lung carcinoma or mesothelioma, and animal studies have failed to convincingly demonstrate the carcinogenicity of talc."

Accidental inhalation of large quantities of talc by infants

A number of reports describe consequences of accidental inhalation of large quantities of baby talc powder by infants. Although the cases do not constitute talc use as intended, they are nevertheless included in this review for the sake of completeness as they present a form of—albeit accidental—talc inhalation.

Brouillette and Weber (1978) describe the case of a prematurely born 1-month-old girl, presented at a hospital in cardiac arrest. She was covered with talc powder which had been poured into her mouth and nose by her 3-year-old brother. Following appropriate treatment of her severe pneumonia, she was discharged after 12 days of hospitalization without apparent consequences. The authors refer in their paper to at least 24 previous cases of massive talc powder inhalation by infants. Most of the children were older than 6 months, and those old enough to play with the powder container were considered at risk. The mortality in these cases was 20%.

Mofenson *et al.* (1981) point out the potential hazard of careless use of baby powder. They reviewed the experience of the Poison Control Center at the Nassau County Medical Center, New York, with baby powder inhalation in children less than 5 years of age, in whom most of the incidents occur. Of approximately 4300 calls in a 6-month period, 40 concerned baby powder inhalation. Symptoms included cough in 14 children, dyspnoea in five, sneezing in five, vomiting in six, and cyanosis in one.

McCormick *et al.* (1982) describe the hazards associated with diaper (nappy) changing, based on statistics from the Massachusetts Poison Control System. Of 138 cases of exposure to various 'poisons' during diaper change in a 3-month period, powders accounted for 47%. Symptoms such as coughing, wheezing and shortness of breath were described as mild, occurring most often with powders.

Motomatsu *et al.* (1979) report the clinical case of two baby girls who died following accidental inhalation of baby powder and unsuccessful treatment. "In order to investigate the harm of baby powder," the authors placed eight mice in a box, the bottom of which was covered with baby powder which was then "blown up" with compressed air. Neither aerosol characterization nor other measurements or experimental data are provided by the authors. Four mice, removed from exposure after 30 and 60 min, "recovered completely". Two other mice, removed after 90 min exposure, died within 6 hours and the last two mice died after 2 hours of exposure. Histopathological findings include haemorrhage, oedema and desquamation of bronchial epithelium.

Pfenninger and D'Apuzzo (1977) describe two cases of powder inhalation. One was a 7.5-month-old girl who had inhaled Fissan baby powder containing talc, zinc oxide and other unspecified substances. The patient responded only slowly to treatment and required 19 days of hospitalization but eventually recovered fully. The second case involved a 13-month-old boy who had inhaled Merfen powder containing talc and borate of phenylmercury. The patient responded well to treatment and recovered completely within 4 days.

De La Rocha and Brown (1989) report a case of baby powder inhalation followed by adult respiratory distress syndrome in a 16-month-old girl who recovered fully after 20 days of hospitalization.

Gutermuth *et al.* (1980) describe accidental talc inhalation and treatment in an 11-month-old female infant and in a 21-month-old boy. The first patient recovered after a 16-day hospital stay, the second one after a 13-day hospital stay.

Mussi *et al.* (1979) report a fatal case of powder inhalation in a 14-month-old girl. After a characteristic fairly asymptomatic initial period—in this case 12 hours—the girl did not respond to treatment and died 41 hours after the accident.

Swanson-Biearman *et al.* (1991) report the case of a 10-month-old boy who was hospitalized for 16 days following massive talc inhalation. The patient had recovered completely by the time of his discharge.

Butenandt *et al.* (1981) treated a 9-month-old male infant who had accidentally inhaled several grams of Penanten powder which contained 96% talc. Prompt bronchial lavage plus appropriate treatment resulted in an asymptomatic status after only 4 days of hospitalization.

Cotton and Davidson (1985) describe the case of a prematurely born 4-month-old male infant with a tracheotomy tube in place to maintain airway patency. During diaper change at home, baby powder was spilled accidentally and apparently blocked the infant's airway. At arrival in the hospital the boy was in cardiac arrest. He was resuscitated but died the following day.

Pairaudeau *et al.* (1991) report respiratory arrest in a 12-week-old boy, following a talc spill on his face during diaper change. He recovered during several days of hospitalization and treatment.

Cruthirds *et al.* (1977) diagnosed progressive diffuse pulmonary fibrosis (talc pneumoconiosis) in a 10-year-old girl who had inhaled a considerable quantity of baby powder at 2 years of age at which time hospitalization was not thought indicated.

Articles by Rumack (1982), Hayden and Sproul (1984), Wagner and Hindi-Alexander (1984) and Hollinger (1990) are mainly brief reviews which do not contribute new scientific information above and beyond the papers reviewed in this report.

Animal studies

Wehner *et al.* (1977c) exposed hamsters to a respirable talc aerosol concentration of approximately 8 mg/m^3 for 3, 30, or 150 minutes/day, 5 days/week for 30 days, or for 30 or 150 minutes/day either until they died naturally or for a maximum of 300 days. The hamsters received cumulative exposures ranging from about 15 to more than 6000 mg/hr/m^3. Estimates based on a pulmonary deposition and clearance study with neutron-activated talc (Wehner *et al.*, 1977b) indicate that 0.05–6 μg talc, depending on the length of exposure, was deposited in the hamster lungs at each exposure. Estimates based on infant-dusting experiments (J. N. Sivertson, personal communication, 1976) show that the weekly hamster exposures, expressed in mg/hr/m^3, exceeded the average weekly infant exposures by some 30 to 1700 times, depending on the hamster exposure group. At death, the lungs, trachea, larynx, liver, one kidney, stomach, uterus, one ovary, or one testis, and all tissues showing gross lesions were collected for histopathological examination. The talc exposures did not affect body weight, survival or the type, incidence or degree of histopathological changes in the exposed groups compared with sham-exposed controls.

Wehner *et al.* (1977b) determined pulmonary deposition, translocation and clearance of inhaled talc in hamsters by a single 2-hour nose-only exposure to neutron-activated talc and subsequent serial killing. Lungs, liver, kidneys, ovaries, skinned carcass and 2-day and 4-day excreta were subjected to γ-ray analysis. The isotope ^{60}Co was used to estimate talc quantities in the samples; the isotope ^{46}Sc was used to check the validity of ^{60}Co as a tracer for talc. From 20 to 80 μg talc (approx. 6–8% of the quantity inhaled) was deposited in the deep lung with a biological half-life of 7–10 days. Alveolar clearance was essentially complete 4 months after exposure. No translocation of talc to liver, kidneys, ovaries or other parts of the body was found.

To validate the interpretation of the pulmonary deposition, translocation and clearance data (Wehner *et al.*, 1977b), Wilkerson *et al.* (1977) investigated whether radionuclides leached from the neutron-activated talc in serum and in dilute hydrochloric acid. Leaching was negligible in both liquids, but somewhat higher in dilute hydrochloric acid than in serum.

A gavage study with neutron-activated talc showed that talc absorption in the gastro-intestinal tract of hamsters is also negligible (Wehner *et al.*, 1977a).

In 1992, the National Toxicology Program (NTP, 1992) prepared for public review and comment a draft technical report on a comprehensive chronic inhalation study conducted at the Lovelace Biomedical and Environmental Research Institute. The study, designed to investigate toxicology and carcinogenesis of talc in rats and mice, followed the standard NTP experimental protocol for chronic inhalation studies. F344/N rats and B6C3F$_1$ mice were exposed for 6 hours/day, 5 days/week to an intended talc aerosol concentration of 0, 6 or 18 mg/m^3. In rats, the exposures resulted in impaired respiratory function; increased lung weights; inflammatory, reparative and proliferative processes in the lungs; hyperplasia of alveolar epithelium; interstitial fibrosis; accumulation of macrophages in lymphoid tissue and regional lymph nodes; and occasionally squamous metaplasia. Incidence and severity of these changes generally were a function of dose. The incidences of alveolar/bronchiolar adenomas and carcinomas were significantly higher in female rats (but not in males) of the 18 mg/m^3 exposure group than in the controls. A significantly increased incidence of phaeochromocytomas of the adrenal medulla in talc-exposed rats of both sexes cannot be explained, as there is no known mechanism by which talc particles deposited in the lungs can affect the adrenal medulla, with the possible exception of a stress-related effect owing to a high pulmonary particle load. In mice, the talc exposures produced chronic inflammation and macrophage accumulation in the lungs, but no hyperplasia, metaplasia or interstitial fibrosis, and no pulmonary neoplasms.

As a relatively recent, comprehensive study that yielded interesting—even puzzling—results, the Lovelace study deserves special comment. Although it was generally well conducted, the study has flaws that can interfere with the interpretation of its results. Inclusion of negative and positive dust control groups would have allowed unambiguous determination of relative toxicity/carcinogenicity of inhaled talc. As it is, the question remains whether the observed pulmonary lesions and other changes in the talc-exposed rodents of the Lovelace study are

talc-specific or a non-specific foreign-body (dust) reaction that is to be expected as a consequence of inhalation exposures at concentrations that result in lung overload.

The investigators were unable to maintain target aerosol concentrations for the 18 mg/m^3 rat exposure group during 19 of the 113–122 weeks of exposure. For 7 of these weeks the rats were exposed to approximately twice the intended aerosol concentration. Even the two intended exposure concentrations led to an impairment of lung clearance mechanisms; both of them, therefore, meet the criteria for a maximum tolerated dose or maximum functionally tolerated dose. On the basis of present knowledge and standards for conducting chronic inhalation studies to investigate carcinogenicity, the chosen talc aerosol concentrations in the Lovelace study were too high. The carcinogenic response observed in female rats of the high-dose exposure group might therefore be attributable to a secondary effect of carcinogenesis, based on a high particle load in the lung (lung overload condition). The Lovelace study can be considered irrelevant for assessing the pulmonary oncogenicity of inhaled talc in humans and the authors of the NTP draft report do not imply any such relevance. Talc aerosol doses received by users of cosmetic talc are several orders of magnitude lower (Aylott *et al.*, 1979; Russell *et al.*, 1979) with no danger of reaching a lung overload condition. The increased incidence of phaeochromocytomas in male and female rats at the high talc exposure concentration remains a perplexing phenomenon that needs to be independently confirmed. Its relevance for humans is questionable, last but not least, again because of the excessive amount of talc accumulating in the rat lungs and the possibility of subsequent stress-related effect. Background incidences of phaeochromocytomas in control rats were already rather high, and no significant increase was observed in the low exposure groups, although these groups also clearly showed symptoms of lung particle overload with impairment of alveolar macrophage clearance function.

The benign pulmonary lesions in the talc-exposed animals generally were those typically observed in inhalation exposures of laboratory rodents to high concentrations of a variety of dusts. There was a significant incidence of bronchiolar/alveolar adenomas and carcinomas in the female rat group exposed to 18 mg/m^3, but not in the males, and not in mice of either sex. The biological significance of this observation remains uncertain.

Pickrell *et al.* (1989) investigated the relationship between the inhalation exposure concentration of talc and the resulting lung burdens and histological lesions. Rats were exposed to 0, 2.3, 4.3 and 17 mg talc/m^3 for 6 hours/day, 5 days/week, for 4 weeks. Lung burdens were 0, 0.07, 0.17 and 0.72 mg talc/g lung, respectively. Mice were exposed to 0, 2.2, 5.7 or 20.4 mg talc/m^3, which resulted in lung burdens of 0, 0.10, 0.29 and 1.0 mg talc/g lung. Histological changes consisted of a modest increase in talc-containing free macrophages within alveolar spaces in both rat and mouse groups exposed to the highest concentration of talc.

Wagner *et al.* (1977) exposed groups of rats to mean respirable concentrations of 11 mg SFA chrysotile or Italian talc/m^3, 7.5 hours/day, 5 days/week, for 3, 6 or 12 months, respectively. Some rats were killed 10 days after termination of exposures, others after one year. Minimal to slight fibrosis as a function of exposure duration occurred in the dust-exposed rats.

In vitro studies

Beck *et al.* (1987) investigated the toxicity of quartz- and asbestos-free talc and of granite (12% quartz), collected from worksites, in hamsters that received the dusts by intratracheal instillation. Dose–response (0.15, 0.75 and 3.75 mg/100 g body weight) and time-course (1–14 days) studies were conducted in bronchoalveolar lavage fluid (BAL). One day after exposure, both talc and granite caused elevated enzyme levels, pulmonary oedema, and increased cell numbers in BAL. Macrophage phagocytosis was inhibited. Response levels were either between 'nontoxic' iron oxide and toxic alpha-quartz or comparable with alpha-quartz. The response to granite dust decreased fairly rapidly as a function of time, but talc exposure resulted in longer elevated enzyme levels and decreased macrophage phagocytosis. The authors conclude that, given similar mass deposition in the lungs, talc causes more lung injury than granite.

Endo-Capron *et al.* (1993) studied genotoxicity of three talc samples in rat pleural mesothelial cells, using genotoxicity assays for unscheduled DNA synthesis (UDS) and sister chromatid exchanges (SCEs). Attapulgite and anatase served as negative controls, chrysotile and crocidolite as positive controls. The positive asbestos controls enhanced UDS or SCEs in treated cultures compared with untreated control cultures, but the talc samples and the negative controls did not.

Davies *et al.* (1983) tested the cytotoxicity of seven specimens of high-purity talc dust in mouse peritoneal macrophages. All samples consistently showed moderate macrophage cytotoxicity, suggesting that they would be fibrogenic *in vivo*.

Talc granulomas from powdered surgical gloves

Talc powder is fibrogenic when administered by various routes to many species of animals (Lord, 1978). When introduced into open wounds it may induce talc granulomas. This phenomenon is used therapeutically in pleurodesis, the deliberate production of adhesions between the parietal and visceral pleura by (usually surgical) deposition of talc or kaolin to treat recurrent pneumothorax. Chappell

et al. (1979) published a survey of the long-term effects of talc and kaolin pleurodesis. There was no increased incidence of lung cancer and no case of mesothelioma in 199 traceable patients who underwent pleurodesis 14 to 40 years previously. Weissberg and Kaufman (1986) successfully used talc for pleurodesis in the treatment of resistant empyema in five patients who completely recovered from empyema with no undesirable side-effects. However, in the vast majority of cases, talc granulomas are undesirable effects of wound contamination with talc, usually from surgical gloves.

Sparrow and Hallam (1991) implicate talc powder in the aetiology of an appreciable number of umbilical granulomas excised from infants and young children. In view of these cases and with reference to the sometimes disastrous consequences of accidental inhalation of baby powder by infants, the authors strongly discourage the routine use of talc powder in the care of infants.

Healey and McDonald (1977) observed a talc granuloma in the right hemiscrotum of a 3-year-old boy who had undergone a right hydrocelectomy 3 months previously. The authors state that the interval for granuloma formation following contamination is extremely variable, ranging from 2 weeks to 45 years, with an average of 2 months after surgery. The extent of granuloma formation depends mainly on the dose and antigenicity of the contaminant and on host response.

Pratt *et al.* (1985) consistently found birefringent particles, which they identified as talc, in the subserosal stroma of hernia sacs. Cellular response was remarkably mild, perhaps owing to the small particle size (about 10 μm) compared with about 50 μm of particles in talc granulomas. The authors further hypothesize that the particles were ingested with medication or food and reached the peritoneal cavity by migration through the intact intestinal wall.

Al-Sheikhli (1978) observed talc powder in granulomas of the vocal cords of a 16-year-old girl and suspects as the cause accidental talc contamination of an endotracheal tube with which she was intubated 4 months previously.

Simsek *et al.* (1992) report a case of severe obstruction of the urinary tract due to a talc granuloma in a 70-year-old male patient 7 years after a suprapubic transvesical prostatectomy.

Pelling and Evans (1986) experimentally tested in rats long-term peritoneal tissue response to mould-release agents and lubricant powder used on surgical gloves. Intraperitoneal implantation of talc produced significantly more adhesions and more severe, persistent, granulomatous reaction than starch powder and calcium carbonate.

Sheikh *et al.* (1984) describe tissue reactions to talc and Keoflo (low cross-linked cornstarch) which they tested as contaminants on the surface of surgical sutures or as pellets implanted in the abdominal muscle of rats. Keoflo caused a strong acute inflammatory reaction which, together with the starch, had essentially disappeared by the fourth week, leaving minimal lesions and scar formation. By contrast, the implanted talc initially induced only mild to moderate acute inflammation followed by chronic inflammatory response, and granuloma formation by the third day. The results suggest that low cross-linked cornstarch is a bioabsorbable substance, whereas talc is not. The authors therefore conclude that low cross-linked cornstarch "is a safe material for use as surgical glove powder", a conclusion not supported by findings of cornstarch-induced lesions reported independently by several other investigators (see below). Kaiser *et al.* (1982) reported similar results with intraperitoneal tests in rats.

Talc may contaminate surgical gloves as a mould-release agent during the manufacturing process or it may be deliberately added as a lubricant for easier donning. In a letter to the editor, Henderson and Griffiths (1979) state: "The large number of talc particles observed on certain American-produced surgeons' gloves, on occasions in excess of 6×10^7 particles/cm^2, would suggest that it is this material that is being employed as the releasing dusting powder in the molding process." Tolbert and Brown (1980) and Khan *et al.* (1983) point out that removal of talc particles from gloves is difficult using recommended washing and wiping procedures, and that a shedding hazard might exist by mechanical dislodging of the particles during surgery.

Rather than reviewing all publications of the last 15 years on talc granulomas, it is the limited objective of this section to show that talc granulomas following surgery can and do occur at various locations and that they can be induced experimentally in animal models. For relatively recent comprehensive reviews of various aspects of glove-related issues, reference is made to the papers by Ellis (1990) and Beck (1992), Fay and Sullivan (1992), White (1992) and Witmeyer (1992). Parenthetically, replacement of talc with cornstarch powder as a glove lubricant has resulted in starch granulomas (Wilson and Garach, 1981), starch peritonitis (Ellis, 1990; Loup *et al.*, 1979; Urdiales Cabal *et al.*, 1989) and intraperitoneal adhesions in rats (Kamffer *et al.*, 1992). With the trend since the 1980s towards powder-free gloves gaining momentum, complications from wound contamination with surgical glove powders may become rarer but not eliminated. To avoid allergic reactions (including anaphylactic shock) in sensitive individuals exposed to water-extractable latex proteins on medical gloves (Beezhold, 1992), vinyl, nitrile, neoprene and copolymer gloves are now available; generally, these gloves are powdered (Witmeyer, 1992).

Ingested talc

Under conditions of normal use, talc can be ingested in one of two ways. First, when used as intended as dusting powder, very small amounts of

talc dust may be inhaled. That portion of the talc particles which is deposited on the ciliated part of the respiratory tract will be transported cranially by the mucociliary escalator mechanism and then swallowed. There are no reports in the literature describing biological effects of these minute quantities of ingested talc. Secondly, talc accounts for the bulk of filler materials in tablets. Appreciable amounts of talc can be ingested with chronic heavy consumption of pills.

Anani *et al.* (1987) describe the case of a 46-year-old male who presented with abdominal pain. Further examination and a right hemicolectomy showed marked fibrosis of the intestinal wall in which birefringent particles with energy-dispersive spectra typical of those for talc were found. The anamnesis revealed that, at the age of 27 years, the patient was treated over a period of 28 months for pulmonary tuberculosis with tablets containing talc (183 g talc per 2670 g total tablet ingestion). The authors speculate that the tablets ingested during this antituberculosis therapy were the source of the talc found in the intestinal fibrosis. If this assumption is correct, the potential consequences of daily multiple pill use, as can be the case with vitamin and mineral supplements, should be more closely examined. Moderate pill consumption does not appear to present a risk. In their review of pharmaceutical excipients, Golightly *et al.* (1988) state, "Ingestion of talc is very unlikely to be toxic."

The Joint Expert Committee of Food Additives of the Food and Agriculture Organisation of the United Nations and the World Health Organization stated in its report on food additives that talc was not mutagenic *in vitro* or *in vivo* and allocated an acceptable daily intake "not specified" classification to food-grade (i.e. free of asbestiform particles) talc (FAO/WHO, 1987).

Discussion

The literature reviewed reflects several areas of concern regarding biological effects of cosmetic talc use.

Hygienic talc use and ovarian cancer

The presence of large numbers of talc particles in normal and diseased ovarian tissue seems indisputable although it is difficult to imagine how inanimate particles without locomotion of their own can breach the formidable cervical barrier and migrate 'upstream' against the ciliary beat of the fallopian epithelium to the ovaries. Several investigators have reported an association between hygienic use of talc and ovarian cancer; none claims to have established a causal relationship. The epidemiological evidence linking hygienic talc use with an increased risk of ovarian cancer generally is weak and sometimes inconsistent: confounding variables were often ignored; the reported increased risk ratios, in most cases less than 2, are barely statistically significant, and epidemiological studies are not sensitive enough to estimate risk ratios less than 2. Talc use might be a causally unrelated marker for confounders associated with increased ovarian cancer risk such as, for example, vulvovaginal disease or obesity. There appears to be a consensus of opinion that more and better designed studies are needed before valid scientific judgement can be passed on whether or not there exists a causal relationship between hygienic talc use and increased ovarian cancer risk.

An interesting question remains. Talc is a recognized fibrogen. If there are sufficient numbers of talc particles in ovarian tissues long enough to cause cancer, where is the ovarian fibrosis which one would expect to develop long before cancer occurs? The only references to ovarian granulomas are those by Mostafa *et al.* (1985) and Kupryjanczyk (1989).

Talc inhalation

In contrast to individuals occupationally exposed to industrial talc, talc pneumoconiosis from personal use of cosmetic talc appears to be extremely rare, occurring only following chronic abuse. When talc, a fibrogenic substance, is chronically deposited at doses sufficiently high to overwhelm the bronchopulmonary clearance mechanism, a fibrotic/ granulomatous tissue response can and will occur, as observed in humans and animals. Animal studies suggest that natural defence mechanisms, such as macrophages and mucociliary clearance, can cope with exposure to talc concentrations considerably exceeding estimated infant exposures, without lesion development (Wehner *et al.*, 1977c). If doses in animal experiments are increased substantially so as to result in pulmonary overload, the results can no longer be considered relevant for human risk assessment. The Lovelace study (NTP, 1992) is a case in point.

Paracelsus recognized some 450 years ago, "*dosis solum venenum facit*" (only the dose makes a poison). Lee *et al.* (1985) confirmed this dramatically by inducing a significant incidence of squamous cell carcinoma in lungs of female CD rats, exposed for up to 104 weeks to 250 mg titanium dioxide/m^3, a substance long considered by many to be biologically inert and frequently used as negative dust control. Should titanium dioxide therefore be considered a carcinogen in rats—and in humans? There is no evidence in the literature to suggest that cosmetic talc, inhaled under conditions of normal use, can cause cancer in the human respiratory system.

Accidental inhalation of baby powder by infants presents a largely avoidable problem if physicians, nurses, parents and other individuals handling the infants are made aware of the potential danger and observe appropriate precautions, such as keeping the powder can out of reach of children. Considering the very large number of dustings administered daily to babies, these incidences fortunately are very rare.

Other studies

In vitro studies have shown that talc is fibrogenic and not genotoxic. The number of papers reporting talc granulomas caused by powdered surgical gloves indicates a certain concern. Calls for talc-free surgical gloves have been voiced. Cornstarch powder does not appear to be a suitable substitute as it, too, causes lesions. The use of talc on surgical gloves presents a special problem that does not affect the typical consumer of cosmetic talc. Ingestion of food-grade talc is unlikely to be toxic.

Conclusion

There is no conclusive evidence in the literature reviewed to indicate that cosmetic talc, when used as intended, presents a health hazard.

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